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PATHOLOGICAL TECHNIQUE

A Practical Manual for Workers in Pathological Histology and Bacteriology

including

Directions for the Performance of Autopsies and for Clinical Diagnosis by Laboratory Methods

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BY

FRANK BURR MALLORY, A.M., M.D.

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General Hospital; Instructor in Pathology, Harvard
University Medical School

Second Edition
Revised and Enlarged
With 137 Illustrations

W. B. SAUNDERS & COMPANY
Philadelphia 1901 London

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TO

HENRY F. SEARS, A. M., M. D.,

WHO BY HIS LIBERALITY FIRST RENDERED POSSIBLE PATHOLOGICAL RESEARCH IN BOSTON, AND BY HIS PERSONAL WORK ADVANCED AND STIMULATED IT,

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THE AUTHORS

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PREFACE TO THE SECOND EDITION.

IN revising the book for this edition we have continued to keep in view the needs of the laboratory worker, whether student, practitioner, or pathologist, for a practical manual of histological and bacteriological methods in the study of pathological material.

The subject of the examination of the urine has been almost entirely omitted, as in the first edition, because it is such a large subject as to require by itself a good-sized volume for its adequate presentation.

In the addition of new methods to the book we have endeavored, as in the first edition, to exercise discrimination, and to select only those that either have stood the test of practical experience or give great promise of usefulness.

The number of illustrations has been considerably increased. The majority are from new photo-micrographs made by Dr. Wright and Mr. L. S. Brown in the Clinico-Pathological Laboratory of the Massachusetts General Hospital. In many instances they replace the illustrations of bacteria in the first edition.

No change has been made in Part I.

In Part II. the sections on Clinical Bacteriology and on Bacteriological Diagnosis have been combined into one section, entitled "Special Bacteriology," for purposes of convenience of reference and avoidance of repetition.

Among many changes and additions may be mentioned the amplification of the description of the Parasite of Actinomycosis and the insertion of descriptions of the Bacillus of Bubonic Plague, of the Parasite of Mycetoma (Madura Foot), and of Wright's methods for the Cultivation of Anaërobic Bacteria.

In Part III., besides numerous minor additions and subtractions which experience and the advance in knowledge have shown to be desirable, there have been added new staining methods for elastic tissue by Weigert, for bone by Schmorl, and for connective tissue by Mallory.

PREFACE.

THIS book is designed especially for practical use in pathological laboratories, both as a guide to beginners and as a source of reference for the advanced. We believe that the book will also meet the wants of practitioners who have more or less opportunity to do general pathological work.

Every autopsy presents for solution a problem which may be simple or complex. The known quantities are certain clinical symptoms and physical signs; the unknown quantities are not only the gross and microscopic lesions that may or may not have given rise to the symptoms and signs, but also the etiology of the lesions and the order of their sequence. The solution of the problem often requires the highest skill in post-mortem, bacteriological, and histological technique, but in its solution lies the fascination of pathological work.

It has seemed advisable to us to present, so far as possible, a consecutive statement of the methods employed in solving the various problems that arise, so as to avoid the repetitions that necessarily occur when the three usual divisions of the subject are separately considered by different writers. It is hoped that this method of presenting the subject will bring the student to the realization that the mechanical performance of a post-mortem examination and the inspection of the gross lesions constitute usually only the beginning of the solution of the problem, which should be investigated bacteriologically, histologically, and chemically as far as our present knowledge will permit.

We should particularly advise the routine bacteriological and histological examination of the more important organs in all suitable cases. Naturally, the autopsies in which the lesions are due to a single etiological factor are the most valuable and instructive for a clear understanding of the pathological processes present.

Besides the methods of post-mortem examinations and of bacteriological and histological investigations connected with autopsies, we have added the special methods employed in clinical bacteriology and pathology.

In the parts devoted to Bacteriology and to Pathological Histology we have not endeavored to make an exhaustive collection of methods and formulæ, but rather to select those which have been found of the greatest service in practical work.

To Dr. A. H. Wentworth, Assistant in Children's Diseases in the Harvard University Medical School, we are indebted for the sections on the Blood and on Malaria, and for the notes in regard to Lumbar Puncture.

BOSTON, August, 1897.

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PATHOLOGICAL TECHNIQUE.

PART I.

POST-MORTEM EXAMINATIONS.

Introduction.—The method of making post-mortem examinations as originally taught by Virchow has been variously modified in its details by his pupils and followers. We have endeavored, while following in general his plan, to select those modifications that have proved simplest and of greatest value. In certain instances we have not hesitated to adopt, or at least to call attention to, useful methods of procedure originating in the Rokitansky school of pathology, as now best exemplified by Chiari.

The problem offered by an autopsy is often solved in part or wholly by the macroscopic post-mortem examination. More frequently, however, the complete and final solution is reached only after careful bacteriological and histological study. The post-mortem examination may, therefore, be looked upon as the beginning of the solution of the problem. Its particular function is to demonstrate in the individual case all congenital or acquired abnormalities, all macroscopic lesions, and to explain all gross mechanical questions. It furnishes the material for bacteriological and histological study. Perfectly to accomplish its purpose a post-mortem examination must be made in a careful, systematic manner.

While a general method of procedure is advisable, it will often be found advantageous, or even necessary, to depart from it. According to Orth, "the chief requisite of every exact post-mortem examination is this, that no part shall be displaced from its position until its relations to the surrounding parts are established, and that no part shall be taken out by whose removal the further examination of other parts is affected."

The order and method of procedure in making a post-mortem examination, including the various incisions, may be said to have been planned for the routine examination of normal or diffusely diseased organs. As soon as a noticeable focal lesion is present the order of procedure and the customary method of removal and of incision must be so altered as best to display the lesion.

Instruments.—The following instruments will be found extremely useful in the autopsy-room, although not all of them are necessary:

The *autopsy-table* should be large, so as to accommodate on it the instruments and several dishes in addition to the body. It should have a slightly raised edge, and should slope gently toward an opening in the center for the escape of fluids. The table is best made of zinc, and along one edge should have a centimeter scale. The water for use on the table is best supplied by a rubber tube from an overhead pipe reaching to within 60 to 100 cm. of the table.

The *scales* for weighing the various organs should have a large pan and gram and kilogram weights.

A *band-saw* will be found very useful for sawing bones for the inspection of the marrow, and for calcified and osseous tumors.

The best *autopsy-knife* is a stout, broad-bladed knife with bellied edge and heavy handle. The blade should measure about 12 cm. in length and 3 cm. in width; the handle should be 12 cm. in length. Many operators prefer a somewhat smaller knife than this.

Amputating-knives of different sizes are useful for long, deep cuts into organs and tumors.

A *myelotome* is a short, thin, narrow knife-blade, 1.4 cm. long and 4 mm. wide, set obliquely on a slender steel stalk ending in a wooden handle (Fig. 2). It is used only for cutting the cord squarely across in removing the brain.

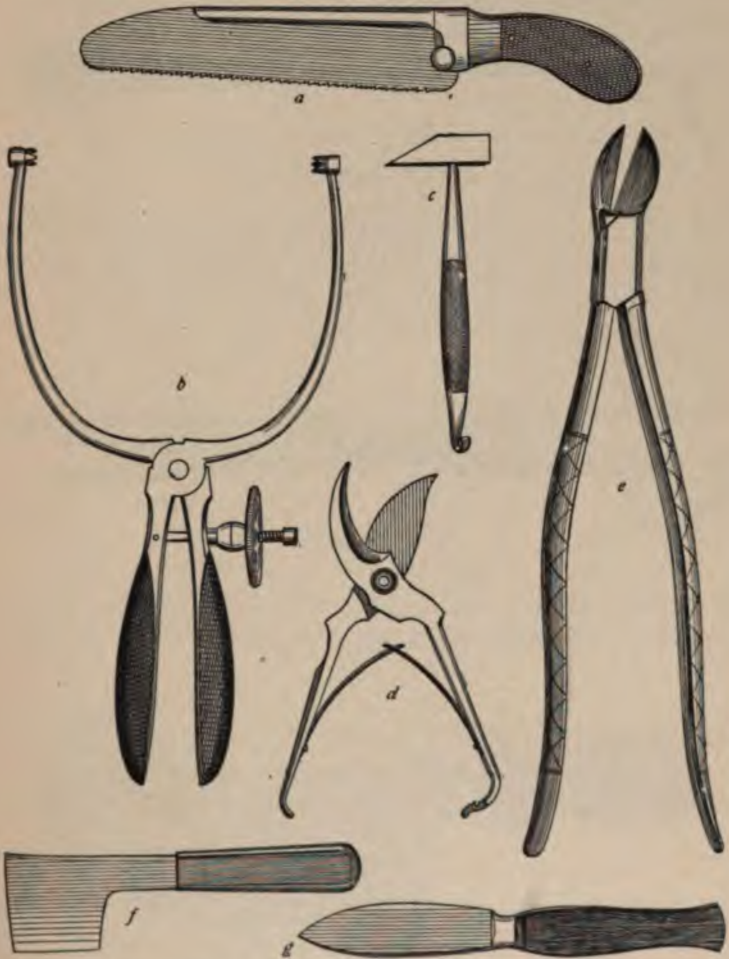

FIG. 1.—Instruments for use in the autopsy-room: *a*, saw; *b*, holder for the head; *c*, steel hammer with wedge end and blunt hook on the handle; *d*, costotome; *e*, bone-cutter; *f*, hatchet-chisel; *g*, autopsy-knife.

Cartilage-knives and *scalpels* of different sizes are useful for a variety of purposes.

Scissors, both straight and curved, should be of various sizes. A medium-sized and a fine pair should each have one probe-pointed blade.

An *enterotome* is a long, straight pair of scissors, of which one blade is longer than the other and blunt at the extremity (Fig. 3). A hook at the end is not advisable. The instrument is used in opening the heart and the intestines.

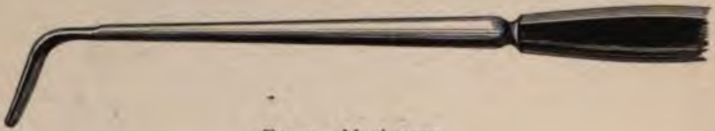

FIG. 2.—Myelotome.

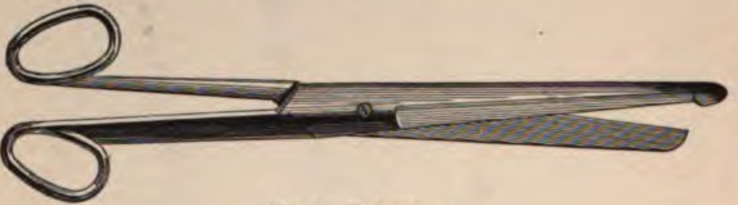

FIG. 3.—Enterotome.

A *saw* with movable back and rounded end will be found the most generally useful for opening the skull and the spinal canal. An ordinary meat-saw is preferred by some, but cannot be used on the vertebræ.

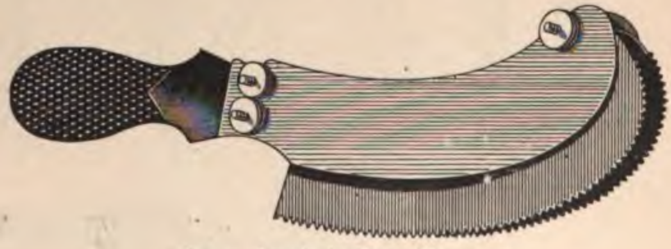

FIG. 4.—Luer's double rachiotome.

Luer's double rachiotome, or adjustable double saw (Fig. 4), is very useful in removing the cord, and is the safest instrument to put into the hands of beginners.

Forceps: several sizes, large and small, mouse-toothed.

Costotome: heavy bone-shears for cutting the ribs.

A powerful *bone-cutter*, with short blades, 5 cm. long, set at an angle of about 45° to the handles, which are 36 cm. in length, is employed for dividing the arches of the cervical vertebræ and for other purposes where ordinary bone-cutters will not do.

A *chisel* with 2 cm. cutting edge, for exposing the marrow of the long bones, removing portions of the base of the skull, etc.

A *hatchet-chisel* of steel for starting the calvaria and spinous processes after sawing the skull and the vertebral column.

Soft-iron hammer with wooden handle.

Steel hammer with wedge end, and blunt hook on the handle.

Holder for the head while sawing the skull.

Autopsy-needles, long and a little curved.

Probes of flexible metal; also fine glass probes for small blood-vessels or ducts.

Grooved director.

Pans for holding water, organs, etc.

Boards, square or oblong, 30 × 30 or 30 × 50 cm., on which to lay instruments or cut organs.

Sponges.

Catheters.

Strong *hemp twine* is the best for sewing up the body.

Glass graduates for measuring fluids.

A *block of wood* with shallow depression for the neck; for use while opening the head.

Vise.

Small *cup* or *dish* for removing fluid from cavities.

General Rules.—The room for an autopsy should be well lighted, otherwise the finer changes in the tissues cannot be recognized. Artificial illumination is not good, because the colors of the tissues are entirely changed by the yellowness of the light.

Before beginning an autopsy the necessary instruments should be arranged on a short board on the autopsy-table in the order in which they are most likely to be used.

The operator stands on the right side of the body. This position he rarely leaves except for some definite purpose; for example, in opening the skull he stands at the head.

Order and cleanliness are the first points to be insisted upon at every autopsy. Clean water should always be at hand for washing the instruments and for keeping the hands free from blood and pus. The cut surface of an organ should not be washed with water except to remove blood; gently scrape the surface with the knife held obliquely.

In cutting, the knife should be drawn, not pressed or shoved into the tissues. According to Virchow, a broad, clean cut into an organ, even if incorrectly made, is much better than several short cuts which leave a ragged surface.

The autopsy-knife should be grasped in the hand as if to cut bread. In using this knife the main movement should be from the shoulder, not from the wrist as in dissecting. It goes without saying that the sharper the knife the better.

In cutting the brain and cord, especially if their consistency is lessened, moisten the knife to prevent the tissue from sticking and tearing.

Before beginning an autopsy it is important to know the main points in the clinical history of the case, as they may greatly lighten the work of investigation by calling attention to those organs that require special examination.

The record of an autopsy should be dictated by the operator as he proceeds with the examination of the case, and should be as nearly as possible an objective description of the appearances found. Only the anatomical diagnosis should express the opinion of the operator. If it is not convenient to dictate the autopsy during its performance, the description of the lesions certainly ought to be made with the organs in sight, and not from memory after the lapse of hours or even days, when many of the details may be forgotten. Later, the results of the bacteriological and histological examinations should be added to the autopsy report, so as to make the case complete.

Rubber gloves are sometimes worn to protect the hands while making a post-mortem examination; but they greatly dull the sense of touch, and cannot be recommended for routine work except in septic cases and while opening the stomach and intestines. Rubber cots for the fingers are often useful.

For cuts on the fingers use celloidin dissolved in equal parts of alcohol and ether, instead of flexible collodion, because the latter will not stick. A cut received during an autopsy should immediately be washed thoroughly, and then sucked so that the blood will flow freely. For protection during the rest of the autopsy use a rubber glove or cover the cut with celloidin.

It is always well to wash the hands after an autopsy with an antiseptic solution, such as corrosive sublimate (1 : 2000). For removing odors from the hands turpentine will often be found serviceable, or a saturated solution of permanganate of potassium followed by oxalic acid. Orth highly recommends a dilute solution of formaldehyde for this purpose.

Suggestions to Beginners.—In a case of *general miliary tuberculosis* the older focus from which the organisms have spread must always be found. Look especially for tubercular thrombi in the pulmonary veins as a frequent source of the general infection.

In a case of *embolism* hunt for the thrombus, bearing in mind, however, that the whole of a thrombus may become free and form an embolus. An arterial embolus may be due to a venous thrombus, in which case it must have passed through an open foramen ovale, except in the case of thrombi of the pulmonary veins.

In *acute peritonitis* always seek for a source of infection (appendix, female genitals, gastro-intestinal tract, etc.). It cannot always be found.

In *hemorrhage from the stomach* associated with cirrhosis of the liver look for rupture of dilated esophageal veins.

In cases of more or less *sudden death*, especially if preceded by signs of asphyxia, always examine the pulmonary artery *in situ* for possible emboli. In cases of *instantaneous death* examine the coronary arteries.

Private autopsies must often be made under many disadvantages, and, when out of town, not infrequently in a short space of time. It is always important to warn the attending physician not to allow the undertaker to inject the body before the autopsy, because the color and consistency of the organs are so changed by most injecting fluids that it is difficult to recognize the pathological processes. If there is danger of post-mortem changes, have the body packed in ice.

A regular autopsy-bag will be found very convenient for carrying to private autopsies. It is made of leather lined with rubber, and measures about 40 × 18 × 20 cm. Loose within it is carried a rubber bag 40 × 24 × 20 cm., shaped like a short envelope with a flap (22 cm. long) on one side, for bringing away any organs that demand further examination. The case of instruments should contain one or two autopsy-knives, two scalpels, a pair of forceps, one or two pairs of scissors, an enterotome, a steel hammer with wedge-end and with a blunt hook on the handle, a small chisel, a saw with detachable handle and back, an autopsy-needle, and a probe; free within the bag should be carried a spool of strong twine, a costotome, a long slender knife for use in removing the brain, a hammer with soft iron head, and a sponge. In rare cases additional instruments may be required. A white duck apron for personal use will always be found convenient. It is also well to carry along several blood-serum tubes and a platinum needle for making cultures at the autopsy. When there is a lesion of the nervous system it is advisable to bring a jar of a 4 per cent. solution of formaldehyde and to place the tissue in the fluid at the autopsy, as otherwise it is not easily gotten to the laboratory in good condition.

At the house can always be obtained a slop-pail, a washbowl, a pitcher of water, several newspapers, and an old sheet. The body is usually on an undertaker's frame, but it may be in an ice-box or on the bed. The examination of the chest and abdomen can be made in any of these positions. If, however, the body is in an ice-box, it must be raised to the level of the top of the box in case it is necessary to open the head.

The clothing on the body can be removed, or, if only a shirt or a night-dress, is best slit down the middle and turned out over the arms. Tear the sheet into four equal pieces. Fold and tuck in one piece on each side of the trunk and neck, allowing the outer portion to fall over the arms. Fold and lay the third piece on the lower extremities, tucking the upper end beneath the clothing below the pubes. The fourth piece can be placed beneath the head if it is to be opened. This procedure leaves the front of the thorax and abdomen free for operation and protects the rest of the body and the clothing. On the thighs place one or two folded newspapers, and on these the necessary instruments. On the legs place the bowl containing only a dampened sponge. If the undertaker has not put a rubber sheet on the floor beneath the body and on the side where the operator is to stand, newspapers should be spread to protect the carpet. Place the slop-pail on the rubber sheet within convenient reach. Having thus made all arrangements, even to the threading of his needle, the operator is ready to begin.

If the cord and brain have to be examined as well as the body, it is best to do the cord first, so as to avoid the leakage that might otherwise occur from the trunk-cavities if they had been opened first. To support the head while opening it, use a stick of wood, a brick, or, in case of necessity, the instrument-box wrapped in a newspaper.

At a private autopsy cleanliness is extremely important. If there is no undertaker or nurse present, the operator himself must see that everything is cleaned and put in order before leaving, that all the blood-stains are removed from the dishes, and that all papers and soiled cloths are burned or rolled up and left in a neat bundle for the undertaker to dispose of. Ground coffee thrown on a shovelful of burning coals will be found helpful in disguising the odor in the room after an autopsy.

EXTERNAL EXAMINATION OF THE BODY.

External examination is often of great importance, especially in medico-legal autopsies, and should never be neglected, as it may throw great light on lesions found within the body. It should be systematic and careful, and is best taken up in the following order:

I. Inspection of the Body as a Whole.

1. Sex.

2. Age.

3. The *body-length* should be measured on the table beside the body, between points opposite the vertex of the head and the sole of the foot beneath the ankle.

4. The *development of the skeleton* has reference to the bony framework, which may be powerful, slender, or deformed.

5. The *general nutrition* is shown by the amount of muscular development and of subcutaneous fat-tissue. The latter is judged by pinching up folds of skin.

6. The *general condition of the skin* includes amount of elasticity, bronzing, jaundice, edema, and decubitus.

7. *Post-mortem discolorations* may be divided into three varieties:

(*a*) *Hypostasis of blood*, or the settling of blood into the lowest lying blood-vessels; this form of discoloration disappears on pressure.

(*b*) *Diffusion of blood-coloring matter* out of the vessels into the surrounding tissues (due to blood-pigment being set free by post-mortem decomposition); does not disappear on pressure.

(*c*) The *greenish discoloration*, usually seen earliest over the abdomen, is due to sulphide of iron formed through decomposition of the tissues. This discoloration is important, as it may modify the interpretation of appearances observed in the internal organs.

8. *Post-mortem rigidity*, degree and extent. It begins in the maxillary muscles, and spreads gradually from above downward, disappearing later in the same order. It is most marked and lasts longest in muscular individuals who have

been ill but a short time. Cholera furnishes the most marked cases. The rigor disappears quickest in cachectic diseases. When once it has been forcibly overcome, it does not recur. The time of beginning after death varies widely—from ten minutes to seven hours.

II. Special Inspection of the Different Parts of the Body.

The examination should begin with the head. Any lesion or abnormality found should be carefully noted. Particular attention should be paid to the condition of the pupils and to the color of the sclera. Then follow in order the neck, the thorax (size and shape), the abdomen (distended or retracted), the genitals, and the extremities.

INTERNAL EXAMINATION OF THE BODY.

The opening of the body-cavities is described first, because the brain is relatively much less frequently the seat of disease, and because in this country it is often impossible to obtain permission to open the head. Moreover, the lesions in the body often throw much light on those to be expected in the brain. The advantage of examining the brain first, particularly in those cases in which the important lesions are cerebral, is said to be that the amount of blood in the cerebral vessels can be more accurately determined. After the heart has been removed some of the blood in the brain may escape through the severed vessels below.

In routine examinations, however, the body is usually examined first, then the brain, and finally the cord. It is not a bad practice to remove the calvarium, to examine the meninges over the upper surface of the cerebrum, and then to make the examination of the body before removing the brain. In this way any change in the blood-supply of the cerebral vessels would be observed.

Opening of the Abdominal Cavity.—In the examination of the body the peritoneal cavity is opened first, the two pleural cavities next, and the pericardial cavity last. The cavities and their contents are to be inspected in the order and at the time that each is opened, but the organs are to be removed from the cavities for further examination in the reverse order, beginning with the heart.

The *primary or long anterior incision to bare the thorax and to open the abdomen* (Fig. 5) should extend from the larynx to the pubes, passing to the left of the umbilicus, so as not to cut the round ligament. In cutting, the handle of the knife is depressed so as to use the belly of the blade rather than the point. An incision beginning as high as the chin is, unfortunately, rarely allowable. Over the sternum the cut should extend down to the bone; over the abdomen, however, only into the muscles, or in fat people through the muscles into the subperitoneal fat-tissue. To open the abdominal cavity, nick carefully through the peritoneum just below the sternum, introduce the first and second fingers of

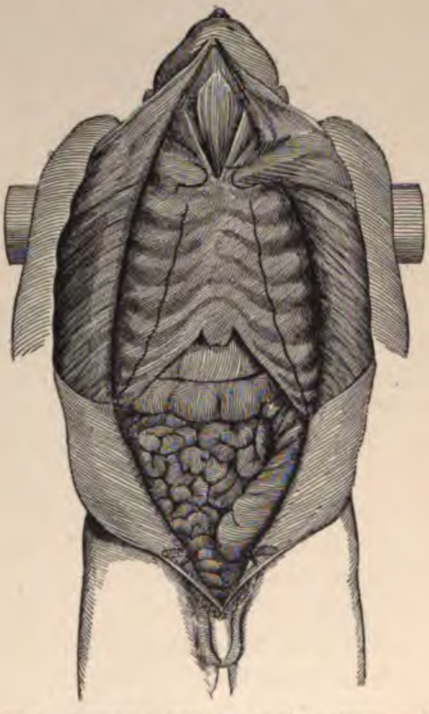

FIG. 5.—Primary incision in the body (Nauwerck).

the left hand, and while making strong upward and outward traction on the right abdominal flap extend the incision to the pubes. Some operators prefer to separate the fingers and to cut between them.

The abdominal flaps are rendered much less tense by cutting the pyramidales and recti muscles from below just above the pubis. Care must be taken not to injure the overlying skin. The abdominal cavity can now be examined, but more room will be obtained if the skin and the underlying muscles be first stripped back from the thorax to about 5 cm. outside of the costochondral line.

The operation is most easily and neatly done by lifting the skin directly away from the chest-wall or turning it forcibly out with the left hand, and then cutting the tense tissue close to the cartilages and ribs with long sweeps of the knife held almost flat. The operation begins over the lower border of the ribs and extends upward. In dissecting off the skin and muscles from the left side the right hand works underneath the left. The mammæ can easily be incised from the under side of the flap, and if necessary the axillary lymph-nodes can be reached by dissecting the skin farther out, especially over the clavicle. Before beginning the inspection of the peritoneal cavity it is important to examine first the surface of the incision into the abdomen, noting the thickness and color of the fat-tissue and the condition of the muscles.

Inspection of the Abdominal Cavity.—The character of any fluid present should be determined and its amount measured or estimated. The simplest way to remove it is to dip it up with a small cup or dish and pour it into a glass graduate for inspection and measurement. If the presence of gas within the peritoneal cavity is suspected, a small pouch should be formed in the first incision as soon as it has been made and water poured in. The first opening into the abdominal cavity should then be made with the point of a scalpel at the bottom of the water, through which the gas, if present, will escape in bubbles.

The various abdominal organs and their relations to each other are to be investigated *in situ* by sight and by touch.

As a rule, examine first the gastro-intestinal tract, including the appendix and the mesenteric lymph-nodes. Ulcerations of the intestine can often readily be made out through the walls. The examination of the spleen, liver, kidneys, and pelvic organs follows. The pancreas is easily reached by tearing through the omentum between the stomach and the colon, so as to open the lesser peritoneal cavity.

After the inspection of the abdominal organs the position of the diaphragm is to be ascertained on both sides in the costochondral line by measuring with the right hand passed palm upward underneath the ribs, and the left hand outside at the corresponding height to mark the position of ribs or intercostal spaces. On the right side the hand is to be passed up on the outside of the falciform ligament. Normally, the diaphragm stands at the fifth rib on the left side, and at the fourth rib or fourth interspace on the right.

Opening of the Thorax.—To open the thorax, cut through the cartilages close to the ribs from the second down (about 5 mm. distant) with a scalpel held nearly horizontal, so that as one cartilage is cut through the handle of the scalpel will strike the next below and prevent the blade from penetrating too far and injuring the lung. In young people the cartilages can be cut easily by one long stroke on each side, but care must be taken not to go too deep. If the intercostal muscles are not divided by the same operation, the sternum can be depressed by the left hand and the muscles severed by one pass of the knife on each side. The lower end of the sternum can now be elevated and freed from below upward from the diaphragm and pericardium until the first rib is reached. The cartilage of this rib is to be cut about 1 cm. farther out than the others, and from below upward toward the clavicle, with the handle of the knife beneath the elevated sternum and with the point and edge of the knife directed upward and a little outward. The sternum is then to be still further freed from the anterior mediastinal tissue until its upper end is reached. The sterno-clavicular joint on the left side can now be easily opened from below by entering a scalpel just above the cartilage of the first rib, and following the irregular line of the joint around the end of the clavicle, while at the same time drawing the sternum over to the right side of the body. The right sterno-clavicular articulation is to be opened by continuing the incision of the scalpel over the upper end of the sternum and into the second joint. The advantage of this method is that there is much less danger of wounding the large vessels at the base of the neck, and thus of mingling blood with any exudation which may happen to be present in the pleural cavities. If preferred, however, the articulations can be opened and the cartilages of the first ribs cut from above before freeing the sternum from the diaphragm. In this case enter a short, sharp, narrow-bladed scalpel held vertically, but loosely, into the left joint on its upper side, starting the incision just outside of the attachment of the sternal end of the sterno-mastoid muscle, and cut around the end of the clavicle by a series of short up-and-down strokes, allowing the blade to follow the irregular line of the joint. After cutting through the joint continue the incision outward and cut through the cartilage of the first rib.

If the cartilages are calcified, use the costotome and cut through the ribs, as more room can be gained in this way, and they are more easily cut than calcified cartilages. When for any reason it is not permitted to open the thorax, the organs within it can be obtained through the opening into the abdominal cavity by freeing the diaphragm from the ribs, and removing first the heart and then the lungs. The sternum should be inspected at the time of its removal. It is perhaps best to examine next, especially in children, the epiphyses of the ribs at the costochondral line for any evidence of thickening.

Inspection of the Pleural Cavities.—In the pleural cavities, as in the peritoneal cavity, the character and amount of any abnormal contents must be determined. If, from the clinical history or from any other reason, the presence of air in a pleural cavity is suspected, a pouch should be formed over the ribs by aid of the skin-flap and filled with water. The pleural cavity is then to be pierced with a scalpel through the bottom of the pouch. Air, if present, will bubble up through the water.

Slight adhesions are best torn through or cut. If the lungs are firmly attached, it is best to strip off the costal layer of the pleura with the lung. This is most easily done by starting the anterior edge of the costal pleura with the handle of the scalpel, and working in first a finger and then the whole hand until the pleura is entirely free. In passing the hand into the pleural cavities protect the back of it, especially if the ribs have been cut through, by folding the skin-flap in over the edge of the ribs.

If desired, the lungs can be drawn forward, examined over their whole extent, even incised, and then replaced until the heart has been removed. In the connective tissue of the *anterior mediastinum* there is almost always a certain amount of emphysema due to the removal of the sternum. Emphysema due to laceration of lung-tissue is more marked in the upper half of the mediastinum, and usually extends up into the neck. The thymus gland attains its full development at the end of the second year, after which time it usually gradually disappears.

Opening of the Pericardium.—To open the pericardium, seize the sac near the middle with fingers or forceps, snip through the wall with knife or scissors, and with either instrument cut upward to where the pericardium is reflected over the large vessels, downward to the lower right border, and lastly to the apex. By gently raising the apex of the heart the amount of fluid in the pericardial cavity can be seen. The normal amount is about a teaspoonful, but it may be increased to 100 c.c. in cases where the death-agony is prolonged. Pericardial adhesions should be broken through with the fingers. If this is impossible, the heart must be incised through the pericardium.

External Inspection of the Heart.—Determine first the position, size, and shape of the heart, and the degree of distention of the different parts. The right ventricle and both auricles are usually distended with blood, which may be fluid as in death from suffocation or more or less coagulated. The left ventricle is contracted and empty unless the individual has died from paralysis of this part of the heart, when it will be found distended with blood (condition of greatest diastole).

Opening of the Heart.—The heart may be opened *in situ* or after removal from the body. Except in certain cases, to be spoken of later, it usually will be found advisable to remove the heart before making any incision into it, for the reason that it can be more perfectly opened after removal, especially by beginners, and the danger of contaminating any bacterial lesions of the valves is lessened.

To *remove the heart*, grasp it gently near the apex with the left hand, supporting it further, if necessary, by one or two fingers placed above the coronal suture, and lift the whole heart vertically upward. Then cut its vessels from below upward with the knife held transverse and oblique. Divide in turn the inferior vena cava, the pulmonary veins on both sides, the superior vena cava, the pulmonary artery, and the aorta. Go deep enough to remove the auricles entire, but avoid injury to the underlying esophagus.

For making the incisions to *open the heart* either a long, slender-bladed knife or long, straight scissors may be used. The heart should be placed on a board with its anterior surface up. The right auricle is opened by cutting from the orifice of the inferior vena cava into that of the superior, and from the latter into the auricular appendage. The first incision to open the right ventricle is made through the tricuspid valve and the wall of the ventricle along the under surface of the right border of the heart. It should be carried to the end of the ventricle, which does not reach quite to the apex of the heart. The second incision begins about the middle of the first, just above the insertion of the anterior papillary muscle (which should not be cut), and is carried through the pulmonary valve well over on the left side along the left border of a narrow, projecting ridge of fat-tissue usually present, so as to pass between the left anterior and the posterior segments of the valve.

The left auricle is opened in a manner similar to the right by incisions joining the four orifices of the pulmonary veins and extending into the auricular appendage.

The first incision into the left ventricle is through the mitral valve along the left border of the heart (*i. e.* the middle of the external wall of the left ventricle), between the two bundles of papillary muscles, to the apex of the heart. The second incision begins at the termination of the first at the apex, and is carried up close to the interventricular septum, parallel to the descending branch of the anterior coronary artery and about 1 cm. from it. The upper portion of the incision should pass midway between the pulmonary valve and the left auricular appendage. Ordinarily, one of the aortic cusps is divided, but this may be avoided, if desired, by dissecting away to some extent the pulmonary artery from the aorta and carrying the incision well over to the right between the right posterior and anterior valve-segments. As each auricle is opened the blood and clots it contains should be carefully removed and the auriculo-ventricular valves carefully inspected from above. In certain cases—as, for instance, extreme stenosis—it may be preferable not to cut through the valve, but to begin the

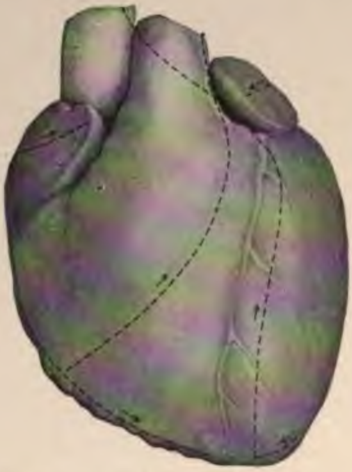

FIG. 6.—Heart, showing incisions.

incision in the ventricular wall below the valve. The ventricular cavities should in like manner be freed from clots and the valves closely inspected. The coronary arteries should always be opened by means of small, narrow-bladed, probe-pointed scissors as far as they can be followed. The examination of the descending branch of the anterior artery is especially important. The posterior coronary is best opened by placing the tip of the left fore finger in the aorta over the orifice of the artery, and cutting from without in toward the finger-tip until the vessel is reached, when it can easily be slit up. In this way injury to the aorta is avoided.

In cases of more or less sudden death with symptoms of asphyxia the pulmonary artery should always be opened *in situ* before removal of the heart, in order to examine for possible emboli, because they often lodge just at the point where the vessels are severed in removing the heart and lungs, and easily may slip out unobserved. The simplest operation is to thrust a sharp-pointed scalpel through the artery just above the valve on the left side in the line of incision already described, and to cut upward until the branches to the right and left lungs are reached. If desired, this incision may be extended down through the pulmonary valve and the ventricular wall along the line given for the second incision in the right ventricle.

The water-test for the competence of the valves of the heart is not very reliable, especially for the auriculo-ventricular valves, and is not so much used as formerly. Inspection and measurement of the valve after the heart has been opened will usually enable one to judge fairly accurately concerning the degree of competence. Before applying the test to the aortic valve the first incision into the left ventricle must be made and the cavity freed from clots, so that no obstruction will exist below the valve. Then the heart is to be held so that the aortic valve is perfectly horizontal, and water poured in from above to float the cusps out. If competent, they should keep the water from flowing through. If, however, in holding the heart the normal relations of the valve and the surrounding parts are not maintained, the valve may leak. A second source of error is that the water may escape through the coronary arteries, branches of which have been cut in opening the ventricle. In testing the mitral valve the left auricle is first opened and the clots removed, so as to expose the upper surface of the valve. Then the nozzle of a syringe is introduced through the aortic valve and water forced in so as to float the mitral curtains up. The test, however, is very unreliable, because the parts cannot be placed under natural conditions.

The pulmonary and tricuspid valves can, of course, be tested by methods similar to those already described.

Increase or diminution in the size of the heart is best determined by weighing the organ after the removal of the clots. In certain cases, however, and in special investigations measurements of different parts of the heart are desirable. Roughly, the heart is the size of the individual's fist.

The following *weights* and *measurements* are taken from *Nauwerck's Sectionstechnik*:

Weight of the heart averages in men,	300 gr.	*Orth.*
" " " " women,	250 "	
Krause gives the average weight of the heart as 292 gr.		
Relative weight of heart to body in men,	1–169	*Krause.*
" " " " women,	1–162	
Length of heart in men,	8.5–9 cm.	*Bizot.*
" " women,	8.0–8.5 "	
Circumference of heart at base of ventricles,	28.8 cm.	(*Sappey*).
Thickness of wall of left ventricle,	1.1–1.4 cm.	*Krause.*
" " right "	0.5–0.7 "	
Thickness of wall of left ventricle (without trabeculæ),	7–10 mm.	*Orth.*
" " right " " "	2–3 "	
Circumference of mitral orifice,	10.4 (W.), 10.9 (M.)	*Krause.*
" " tricuspid "	12.0 (W.), 12.7 (M.)	
" " aortic "	7.7 (W.), 8.0 (M.)	
" " pulmonary orifice,	8.9 (W.), 9.2 (M.)	
" " ascending aorta,	7.4 cm.	
" " pulmonary artery,	8.0 cm.	(*Buhl*).

The directions given for the removal and opening of the heart apply only when the organ is normal or contains lesions within itself which are not in continuity with any of the vessels entering into it. In aneurysm of the ascending aorta, in thrombosis of a vena cava, and in a number of different lesions connected with the heart or with the vessels given off from it, it is important to examine these vessels and to open them while they are still in continuity with the heart. For this purpose it is often necessary or advantageous to remove the thoracic organs in one piece, so as to be able to examine the central circulatory apparatus in continuity from the front and back before disturbing any of its relations. This is done by cutting across the trachea and adjoining tissues as high in the neck as necessary or possible, and dissecting them free from the cervical vertebræ and the first ribs. Then by drawing the trachea and surrounding tissues forcibly forward the aorta and overlying organs can be easily stripped from the vertebral column as low as the diaphragm. The left hand is now placed around the lower end of the pericardial sac, the aorta, and the esophagus just above the diaphragm, and the vessels are severed by cutting between the hand and the diaphragm.

More space for the examination *in situ* of the vessels at the base of the neck can be obtained by freeing the clavicles from all attachments above and to the first ribs and drawing them forcibly outward; this operation will be found especially useful in following up the subclavian vessels.

Removal of the Lungs.—Pleural adhesions have already been spoken of. If the base of the lung is adherent to the diaphragm, it is usually advisable to remove the latter with the lung by cutting through its insertion into the ribs. According to Orth, there is less danger of wounding the abdominal organs if scissors be used for the performance of the operation. After the lung is free it is drawn forward out of the pleural cavity, and the root of it is grasped from above downward between the separated fingers (first and second or second and third) of the left hand. The lung, thus resting in the palm of the left hand, is first drawn downward toward the pubes until the primary bronchus is divided by a nearly vertical incision above and behind the left hand. Then the lung is lifted vertically upward, and the rest of its attachments cut in the same direction from above downward by the knife held transverse and flat, so as to avoid injuring the esophagus and aorta.

The procedure is the same for both lungs. Once in a great while the apex of a lung will be found so firmly adherent by dense scar-tissue that it can be freed only by using the knife.

The primary or main incision into a lung is a long, deep cut from the apex to the base and from the convex surface to the root, slitting the primary bronchus, and thus not cutting it off from its branches to the upper and lower lobes (Fig. 7). To incise the left lung, place it with its inner or median surface and root downward on a board and with its base toward the operator. The left thumb steadies the lower lobe; the first finger reaches between the two lobes almost to the primary bronchus; and the rest of the fingers should hold the upper lobe.

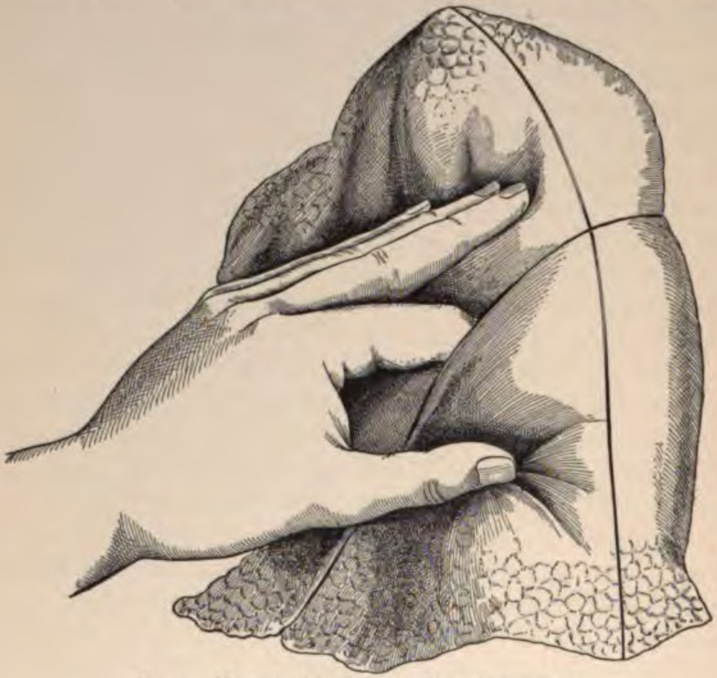

FIG. 7.—Method of incising the lung (Nauwerck).

The right lung is most easily incised by placing it in the same position, but with the apex toward the operator; in other words, always place the anterior edge of a lung beneath the palm of the hand. Some prefer to place each lung on its lower or diaphragmatic surface for incision. The right middle lobe is incised separately by a cut extending transversely in its greatest diameter.

The bronchi and blood-vessels should be opened up for some distance with small probe-pointed scissors—as a rule from the surface of the section—cutting through the overlying lung-tissue. In some cases, however, it is best to open up both the blood-vessels and the bronchi from the outside of the lung before incising it. The order to follow is vein first, then artery, and finally the bronchus.

Secondary cuts into the lung are to be made parallel to the main incision.

The bronchial lymph-nodes should be incised from the outside of the lung.

Organs of the Neck.—The operation of the removal of the organs of the neck is greatly facilitated if it is possible to continue the primary skin-incision up to the chin. In other cases dissect the skin from the larynx and muscles of the neck as far up as possible. In like manner free the muscles, esophagus, and trachea from their attachments laterally and posteriorly. Then allow the head to drop well back over the end of the table, and pass a long, slender-bladed knife up between the skin and the larynx, just behind the symphysis of the lower jaw, until the point of the knife appears beneath the tip of the tongue. From this point the knife is carried with a sawing motion down first one ramus of the jaw and then the other, dividing laterally the glossal muscles as far back as the posterior pharynx. The knife is next carried up behind the esophagus, and the posterior wall of the pharynx divided as high as possible. Pass the left hand up inside of the neck and draw down the tongue. Then cut the attachments of the soft to the hard palate, carrying the knife well out so as not to injure the tonsils. Any remaining attachments are usually easily severed by pressing the tongue first to one side and then to the other, and cutting close to the roof of the pharynx.

Each lobe of the thyroid gland is to be incised in its greatest diameter.

Next cut through the middle of the uvula and examine all of the pharynx removed. Incise the tonsils vertically. The esophagus is to be slit in the median line posteriorly; if it is normal, the larynx and trachea are then slit in the posterior median line also, thus splitting the esophagus in two.

The Abdominal Cavity.—The order of removal of the abdominal organs varies with different operators, and under varying circumstances with the same operator. The gastro-intestinal tract, including the liver and pancreas, may be removed before or after the genito-urinary tract. The spleen as an organ by itself is often the first to be removed. The early removal of the liver is occasionally advantageous for the sake of the additional space obtained for the examination of the other organs. It is well to practise the different methods of procedure, so that in a difficult case the best may be selected, because the examination of the abdominal cavity, especially in cases of extensive disease with numerous adhesions, is often one of the hardest tasks in post-mortem technique. As a rule, it is best to follow the usual order as long as possible, gradually removing the more or less normal or uninvolved organs. Occasionally it may be advisable to remove the organs *en bloc*, so as to be able to approach the problem from all sides.

In all cases of acute peritonitis it is best before removing any organ to search for the source of the infection, paying particular attention to the vermiform appendix, to the gastro-intestinal tract, and, in females, to the pelvic organs.

The order of removal of the abdominal organs adopted in this book for the majority of cases is that which seems the simplest and most natural—namely, to remove first the spleen as an organ essentially by itself; secondly, the gastro-intestinal tract, including the pancreas and liver, which forms the upper layer; thirdly, the genito-urinary tract or middle layer, leaving the circulatory tract, the lowest layer, to be opened and inspected *in situ*. If, however, it proves necessary to open a part of the gastro-intestinal tract *in situ*, it will be neater perhaps to remove the kidneys and spleen first. Occasionally at private autopsies it may be unnecessary to examine the intestinal tract; under these circumstances it is important to be able to get at the different organs without taking out the intestines.

The Spleen.—As a rule, the spleen can easily be drawn forward from its bed behind the fundus of the stomach, beneath the diaphragm, and lifted on to the lower edge of the ribs on the left side without cutting its vessels. The organ is then to be incised in its greatest diameter while thus firmly fixed between the left hand and the ribs; or the vessels may be cut close to the hilus and the spleen incised after being placed on a board.

In cases of adhesion to the diaphragm the spleen must be handled carefully while the fibrous attachments are torn or cut through, for the capsule is easily ruptured. Occasionally it is advisable to cut out with the spleen the portion of diaphragm attached to it.

The important anatomical structures to be noted in the macroscopic examination are the capsule, trabeculæ, blood-vessels, lymph-nodules, and pulp. The weight of the spleen, according to Orth, varies from 150 to 250 grams. The average weight is put at 171 grams. The spleen measures 12 × 7.5 × 3 cm.

The Gastro-intestinal Tract.—The first step is to examine externally, more or less carefully according to the clinical symptoms, the whole tract from the stomach to the rectum, if it has not already been done at the primary inspection of the peritoneal cavity. The main points to notice are distention or contraction of the intestines, injection of the blood-vessels, thickening of the wall, especially in the lower part of the ileum, adhesions, exudations, etc. Inspect the mesentery, its length, the amount of fat, and the size of the lymph-nodes; incise the latter to determine color and consistency. Examine the mesenteric vessels if any evidence of infarction of the intestine is noticed. The portal vein and its branches should be opened up *in situ*, in all cases of abscess of the liver or of secondary deposits in it of malignant growths, before the gastro-intestinal tract is removed. As a rule, it is not necessary to open any part of the gastro-intestinal tract *in situ*. The operation can be performed much more neatly at the sink. The duodenum is often opened for the sake of investigating the flow of bile from the gall-duct, but except in cases of jaundice the operation must be looked upon largely as a physiological experiment.

Free the *omentum* from the transverse colon by putting it on the stretch and dividing it with the knife close to the colon. Then begin the removal of the large intestine by drawing the sigmoid flexure forcibly forward and cutting the mesocolon close to the gut, first down to the rectum, then upward to the transverse colon. Free the latter by dividing the two folds of the lesser omentum, if not already cut through, which unite it to the stomach. The ascending colon is to be freed in the same manner as the descending portion. Care should be taken not to injure the appendix. If the lower part of the sigmoid flexure be now stripped upward a short distance with the fingers, so as to force the intestinal contents out of the way, the gut can be divided just above the rectum without fear of the feces escaping.

Place the freed intestine in a pan or pail, and as the small intestine is divided from its mesentery deposit it in the same receptacle. To remove the small intestine, begin at the cecum, and, while lifting the ileum with the left hand strongly enough to keep the mesentery constantly tense, cut the latter close to the intestine by playing the knife easily backward and forward across it with a fiddle-bow movement. Continue the operation until the duodenum is reached. The mesentery can now be dissected from the duodenum and removed, or the mesentery, duodenum, pancreas, and stomach can be removed in continuity with the intestine by carefully dissecting them off the underlying structures. The operation is perhaps more easily accomplished by freeing the organs from below upward. First cut down through the diaphragm and free it around the esophagus. Then separate the stomach from the liver by means of the thumb and fingers of the left hand in such a way as to put on the stretch the vessels of the hepato-duodenal ligament. These vessels (hepatic artery, common gall-duct, and portal vein) are then carefully divided in the order named. As each vessel is cut the character of its contents should be observed to see if anything abnormal is present.

The mesentery, if still present, the duodenum, the pancreas, and the stomach, are now to be dissected carefully away from the underlying vessels from below upward until the esophagus is reached. This may be constricted by the fingers at any point desired, and cut across without danger of the gastric contents escaping and without the necessity of tying. In certain cases of hemorrhage from the stomach associated with cirrhosis of the liver it is important to remove the esophagus in continuity with the stomach, because in these cases the hemorrhage usually takes place from dilated esophageal veins.

The *stomach* and *intestines* are now to be opened at the sink by means of the enterotome, the colon along one of its longitudinal muscular bands, the small intestine along its mesenteric attachment, because the most important lesions usually occur opposite this line in the lymph-nodules and Peyer's patches. The stomach is opened by many along the greater curvature; others, however, prefer to cut along a line 3 cm. from the lesser curvature, on the ground that better museum preparations are thus obtained. In case any tumor or focal lesion is perceived from the outside, it is advisable to cut the stomach, if possible, in such a way as to leave the pathological part uninjured.

Whenever jaundice is present the duodenum must be opened *in situ* in order to examine the bile apparatus in continuity, so as to determine whether the coloring is due to obstruction of the hepatic or common gall-ducts, or is of so-called hematogenous origin.

To open the *duodenum* make a transverse fold in the anterior wall and incise with the scissors. Continue the longitudinal slit thus made up as far as the pylorus and down to where the duodenum passes beneath the mesentery. Notice the contents of the duodenum and their color both above and below the opening of the gall-duct. The ductus choledochus usually opens in common with the ductus pancreaticus on the posterior wall of the duodenum a little below the middle of the head of the pancreas, at a point marked by a small papilla which can easily be recognized by putting the mucous membrane on the stretch transversely. Press first on the common duct gently and in the direction of the papilla, watching the opening to see if any obstructing material is forced out. Pressure is then to be made on the gall-bladder to see if its contents also will flow. If necessary, the common duct and its branches are to be opened *in situ*. In certain cases the ductus pancreaticus is likewise to be opened up.

Several cross-sections of the *pancreas* are usually better than one in the greatest diameter, because the duct is left in a better condition for slitting up if necessary. The weight of the pancreas varies from 90 to 120 grams (Orth). It measures 23 × 4.5 × 2.8 cm.

The Liver.—The liver is usually the last organ of the gastro-intestinal tract to be removed. This is ordinarily done by lifting up the right lobe and freeing it from all attachments as far as the vertebral column: the right lobe is then lifted and placed on the edge of the ribs on the right side, while the left lobe is elevated and freed. If the diaphragm is firmly adherent, remove it with the liver. The incision to display the liver is a long deep cut passing through the right and left lobes in the greatest diameter of the organ.

In a good many cases it is very convenient to remove the liver at the beginning of the special examination of the abdominal cavity, because more room can be obtained for the investigation of the other organs. This latter fault can to some extent be obviated by cutting the diaphragm on the right side and allowing the liver to slide forward somewhat into the right thoracic cavity.

There can be no objection to the removal of the liver when jaundice is not present or when the liver is not connected by continuity with the lesion of some other organ (pylephlebitis, malignant growth extending through portal vein or along gall-ducts, etc.).

The operation is performed as follows: Pass the left hand in between the diaphragm and the right lobe and push the liver forward out of the right hypochondrium. Incise it deeply in its greatest diameter through the left and right lobes. Next free the gall-bladder from its bed by means of the fingers, and cut it off near the ductus hepaticus after compressing its lower end. It can then be opened lengthwise and washed without danger of discoloring the liver or other organs. The liver is now to be grasped by placing the thumb on the under surface of the liver and the fingers in the incision. Elevate the organ, and, while carefully watching, cut through the hepato-duodenal ligament, which includes the blood-vessels and the ductus hepaticus. The ligamentum hepato-gastrium, the inferior vena cava, the suspensory ligament, the ligamentum coronarium, and the tissue between the inferior surface of the liver and the upper end of the kidney follow next: the adrenal is to be left on the kidney, and the diaphragm ought not to be injured.

Even in the ordinary way of removing the liver the organ will be found much easier to handle if the usual incision is made *in situ*, so as to furnish a hold for the left hand.

Other cuts into the liver are best made parallel to the primary one.

Orth gives the weight of the liver for adults as varying from 1000 to 2000 grams. The average weight is usually put at 1500 to 1800 grams.

The liver measurements are as follows:

Length from right to left	25–32	cm.
Width of right lobe	18–20	"
Width of left lobe	8–10	"
Vertical diameter of right lobe	20–22	"
Vertical diameter of left lobe	15–16	"
Greatest thickness	6– 9.5	"

The Kidneys and Adrenals.—If the adrenals are to be removed with the kidneys, it is necessary to cut first to the inside, and secondly above the adrenal, and then to make from the outer end of the second cut a curved incision along the outer convex border of the kidney through the peritoneum and the perinephritic fat-tissue. The left hand is to be inserted into the cut, the mass of tissue drawn forcibly forward, and the vessels divided as close to the aorta as possible, so that the renal vessels may be slit up and examined in connection with the renal lesions. The adrenal should be incised crosswise. The kidney is to be held firmly in the left hand between the thumb and fingers while a longitudinal incision is made from the convex border to the hilus. As a rule, it is better to shell it out of its investing fat-tissue before incising it.

It will often be found convenient to make simply the curved incision above given, to shell the kidney out of its fat-capsule, and then to divide its vessels, leaving the adrenal behind to be incised *in situ* or removed separately. As a rule the left kidney is removed first.

In all cases in which the bladder is involved in pathological changes in common with the kidneys the whole urinary tract should be removed intact, so that the lesions may be examined in continuity. For this reason it is a good plan to open up the pelvis of the kidney and the ureter from the primary incision, in order to see if any lesion is present before dividing the ureter.

If it is desired to remove the kidneys before the intestines, the latter must to some extent be freed from their normal attachments.

The splenic flexure of the colon is first to be drawn forcibly forward and its attachments divided where they hide the left kidney. If the ureter is to be taken out also, it is best to free the whole of the descending colon from its mesocolon. Then the colon and the coils of small intestine are drawn over to the right side of the body, so as to leave the left kidney and adrenal exposed. They are then removed in exactly the same manner as already described.

To remove the right kidney the hepatic flexure must be freed from over it. If the ureter is to be taken out, the descending colon and the cecum are dissected from over it.

The right adrenal is firmly attached to the under surface of the liver, and must be carefully dissected from it by turning the latter upward.

If the urinary tract is to be removed in continuity, each ureter is dissected down to the brim of the pelvis, and then left with its kidney attached until the pelvic organs have been taken out.

After the kidney has been incised the capsule is to be stripped off, at least in part, so that the appearance of the surface of the kidney and the presence or absence of adhesions between the capsule and the renal tissue can be determined.

The points to be noted in the macroscopic examination of the kidney are size, consistency, and, on section, color, relative proportion of cortex to pyramids, and thickness of each; finally, the normal markings of the kidney, including blood-vessels, glomeruli, convoluted and straight tubules of cortex, collecting tubules of pyramids.

The average weight of the kidney is 150 grams. The left kidney is always 5 to 7 grams heavier than the right (Orth). A kidney measures 11–12×5–6×3–4.5 cm. The cortex measures in thickness 4–6 mm. The relation of the cortex to the medulla is 1 to 3.

The Pelvic Organs.—The pelvic organs are most easily and neatly removed by stripping the peritoneum from the pelvic wall with the fingers. Begin over the bladder and extend down the sides of the pelvis until the fingers meet beneath the rectum. Brace the backs of the hands laterally on the brim of the pelvis and lift the fingers forcibly upward; this movement will free the pelvic organs cleanly from the sacrum, and leave them attached only anteriorly at the rectal and genital openings, and posteriorly by the peritoneum and the vessels at the brim of the pelvis.

Anteriorly, the attachments may now be divided with the knife at whatever point seems advisable, ordinarily close to the pubes just anterior to the prostate (or through the urethra and vagina in females) and through the lower end of the rectum. Posteriorly, cut through the tissues at the brim of the pelvis, taking care not to cut the ureters if the kidneys are still attached to them. The *rectum* is to be opened with the enterotome along the posterior wall, and the inner surface thoroughly washed off so as to avoid soiling the other organs.

To open the *bladder* in males, especially if the penis has been removed in continuity with it, incise with the scissors a transverse fold in the anterior wall of the fundus, and carry the incision through the urethra and along the dorsum of the penis. To accomplish the latter act perfectly the *penis* must be firmly stretched by having an assistant pull at the frenum while the bladder is held fixed by the operator.

In females it is usual to enter the scissors into the bladder through the urethra and to cut through the middle of the anterior wall of the fundus.

In males the *rectum* should be dissected from the bladder, so as to lay bare the vesiculæ seminales and the prostate, which are examined by means of several transverse incisions.

In females, if the bladder is normal, the *vagina* is incised in the anterior wall through the middle of the bladder. Or the vagina may be incised laterally until the cervix is reached, and then the cut be carried up to the median line.

The *uterus* is incised in its anterior wall from the cervix to the fundus. From the upper part of this incision secondary incisions are carried out on each side to the orifices of the Fallopian tubes.

The *ovaries* are incised in their greatest diameter, from the convex border to the hilus. Weight of ovaries, 7 grams.

The *testicles* can readily be examined without external injury to the scrotum by cutting underneath the skin over the pubes down to the scrotum on either side of the penis, and shoving the testicles up through the incision. Cut carefully through the overlying tissues until the cavity of the tunica vaginalis is opened. Remove the testicle by severing the cord. The incision to display a testicle should be in the long diameter, beginning on the side opposite the epididymis and extending through into it. Weight of testicles, 15–24.5 grams. In cases of tuberculosis of the testis and epididymis it is advisable not to cut through the cord, but to remove the testicles and cords with the bladder, so that the whole genital tract may be examined in continuity and the associated lesions in the vesiculæ seminales demonstrated, if present.

The *penis*, or at least the larger portion of it, can be removed in connection with the bladder by continuing the primary body-incision out to about the middle of the dorsum of the penis, which is then to be freed from the investing skin and divided just posterior to the corona. It is next dissected back to the pubic arch, and freed from it partly by cutting from without, partly from within, the pelvis, until the penis can be passed underneath the arch into the pelvis. Other methods are to cut through the symphysis, which can then readily be sprung apart by swinging one of the legs out in a horizontal plane, or even to saw out a small section of bone including the symphysis, so as to have more room for freeing the attachment of the penis and for removing it.

The structures now remaining in the abdominal and thoracic cavities which require examination are the large blood-vessels, the thoracic duct, the celiac ganglion, and the retroperitoneal lymph-nodes. The *inferior vena cava* and its branches are first examined (especially in all cases of pulmonary embolism) by slitting them with scissors along the anterior wall. If it is necessary to follow the iliac vessels into the thigh, it will be found easier in sewing up if the primary abdominal incision is continued off to the side in question, thus giving a single though curved incision.

It is sometimes advisable to open up the inferior vena cava and its branches before removing the pelvic organs, so that thrombi extending into the pelvic vessels may be examined before they are disturbed.

The *semilunar ganglia* lie on the aorta, around the celiac axis, above the pancreas.

The *thoracic duct* lies behind and to the right of the aorta. In the thorax it is most easily found by dissecting on the right side between the aorta and the azygos vein. The receptaculum chyli lies to the right and behind the aorta upon the second or third lumbar vertebra. Examination of the thoracic duct is of especial importance in cases of tuberculosis of the intestine and mesenteric lymph-nodes with secondary miliary tuberculosis.

The *aorta* is to be opened *in situ* along the anterior wall throughout its whole extent, and the iliacs as far as the femoral ring.

Besides the brain, the spinal cord, and the thoracic and abdominal organs, it is often necessary to examine or remove for study other portions of the body that are affected by disease. A little ingenuity will enable one in appropriate cases to get at almost any part desired.

A view of the marrow in a long bone is most easily obtained in the femur by extending the body-incision down over one of the thighs, dissecting the muscles way, and then chiselling off a portion of the upper part of the shaft.

In tuberculosis of the spine it is quite easy to remove any part, or even the whole, of the vertebral column, including the pelvis and portions of the femurs, without other incisions than the one from the neck to the pubes, with extension down the thighs in case parts of the femurs are to be taken out. Divide the ribs a few centimeters from the vertebral column on each side of the portion that is to be removed, cut through intervertebral disks both above and below it, and then carefully dissect it free, taking great care not to button-hole the skin.

Removal of the Brain.—The incision into the scalp should begin from one to two centimeters behind the right ear, near its lower border, at the edge of the hair, and extend over the vertex of the skull to a corresponding point behind the left ear. The cut is most easily made by thrusting a small narrow-bladed scalpel, with its back toward the calvarium and its point toward the vertex, through the skin behind the ear and shoving it along in the desired direction. By making the incision in this manner the hair is not cut, but simply parted. The anterior flap should be stripped from the calvarium and the temporal muscles by putting it on the stretch and dividing the loose connective tissue holding it by sweeping strokes of the scalpel nearly as far forward as the orbits. After a part of the flap has been freed it is often possible to strip the rest without using the scalpel. For the posterior flap, which should be removed back as far as the occipital protuberance, the scalpel nearly always has to be used.

If the hair is long, the anterior portion can be rolled into the anterior flap over the face and thus protected. The posterior portion is gathered at the nape of the neck, and then a towel is wrapped tightly around the head and neck, extending from the line where the flaps are reflected down to the shoulders, and is pinned over the lower part of the forehead. In this manner the hair is perfectly protected from being soiled and ample room is left for work.

Of the two methods of opening the skull, the circular and the wedge-shaped, the former makes the better museum preparation, but the latter is in greater use in this country, and has the advantage of rendering the calvarium less likely to slip out of place after the head has been sewed up.

The wedge-shaped incision consists of three cuts, which should be outlined on the periosteum of the skull with a scalpel. The first cut begins just above and behind the left ear, and is carried over the forehead just back of the edge of the hair or over the frontal eminences to a corresponding point above and behind the right ear. The two other cuts begin at each end of the first incision, forming there an obtuse angle, and are carried back to meet in the median line behind at an angle of about 160° a little in front of the occipital protuberance. The temporal muscle on each side is now to be scraped back from the line of incision out of the way of the saw, but is not to be cut off. The holder, if one is used, is attached with a foot in each obtuse angle in the temporal region. If a holder is not employed, the head is best steadied by hands on the calvarium and face. Use towels or cloth to prevent slipping.

Start the incision with the saw over the forehead and extend it back along the line marked out. It is best not to carry the incision clear through the inner table of the bone, for two reasons: first, on account of the danger of injuring the brain-substance; secondly, because if the inner table or a part of it is cracked through with a chisel and hammer, it can be done without injuring the underlying tissue, and the irregular overlapping fragments of bone thereby formed serve afterward for holding the calvarium firmly and steadily in place.

After sawing along the lines marked out, insert a chisel in the frontal region, and with a quick, sharp blow crack through the rest of the inner table. In like manner insert the chisel in the middle of the other incisions and free the calvarium posteriorly. To remove the calvarium insert the chisel end of the hammer in the incision in the frontal region, and press down with the left hand while swinging the handle around in a horizontal plane.

By means of the powerful purchase obtained the calvarium is easily started. Then catch the hook of the hammer over the calvarium and strip it off. If the dura is adherent to the calvarium, it may be freed by using the point of the closed enterotome to pry it off.

In young children, and sometimes in old people, it is necessary to remove the dura with the calvarium. To do this, cut through the dura with the point of a scalpel along the lines of incision in the skull; then cut the falx cerebri in the median line, both anteriorly and posteriorly.

An infant's skull is best opened by cutting with a pair of scissors through the dura along the sutures (in the longitudinal suture on each side of the falx) well down to the floor of the skull. This gives five bone-flaps which may be turned out like the petals of a flower, leaving the brain uninjured. It is often necessary to cut half of the base of each flap in a horizontal line to aid its being turned out. The falx cerebri must of course be divided anteriorly and drawn back before the brain is removed. In sewing up, the bone-flaps are turned in over a bag of sand or sawdust filling the cranial cavity, and are kept perfectly in place by the skin.

In a case of fracture of the skull no cracking with hammer and chisel is allowable; the calvarium must be freed entirely by sawing. The calvarium should be examined at the time of removal.

The next step is to inspect the dura. Under normal conditions it is not tense in the frontal region, but can be picked up with the forceps or fingers. If the dura is not thickened, the convolutions normally should be visible through it. The longitudinal sinus is opened with knife or scissors and its contents examined. Pacchionian granulations are not infrequently found projecting into it.

To remove the dura, cut through it with scissors or knife along the same lines in which the calvarium was sawn. Turn back each half of the dura and examine the surface of the convolutions and the inner surface of the dura. The convolutions should be distinct and rounded, not flattened, with obliteration of the gyri, as occurs when there is internal pressure.

The Pacchionian granulations are situated along the longitudinal fissure and may grow through the dura and form depressions in the calvarium. There may be apparent adhesions between the dura and pia due to veins passing from one to the other. The dura is still further freed by seizing the two halves anteriorly and lifting them up until the falx is tense at its insertion into the crista galli. Pass a knife in parallel to the falx, on the left side, with the edge forward, as far as the cribriform plate; turn it to the right and cut until the falx yields. Withdraw the knife in the same manner in which it was inserted. Next draw the dura back. It is usually more or less attached along the longitudinal fissure by Pacchionian granulations and by blood-vessels. These may be cut or torn through. Do not cut the dura posteriorly, but let it hang down.

To remove the brain, insert the two fore fingers, or the first and second fingers of the left hand, anteriorly between the dura and the frontal lobes, one on each side of the falx cerebri, and draw the brain gently back until the optic nerves are visible. Ordinarily, the olfactory nerves come away from the cribriform plate without trouble, but sometimes have to be freed with the point of the knife. With a long, slender-bladed knife divide the optic nerves as far forward as possible while holding the brain back with the left hand. Continue to draw the brain carefully back and divide the cranial nerves and the carotids. Then draw forward first the left, then the right temporal lobe, and cut the tentorium close to its attachment to the petrous portion of the temporal bone with a sawing motion, using the tip of the knife. Insert the knife at the side close to the squamous bone, and cut from there

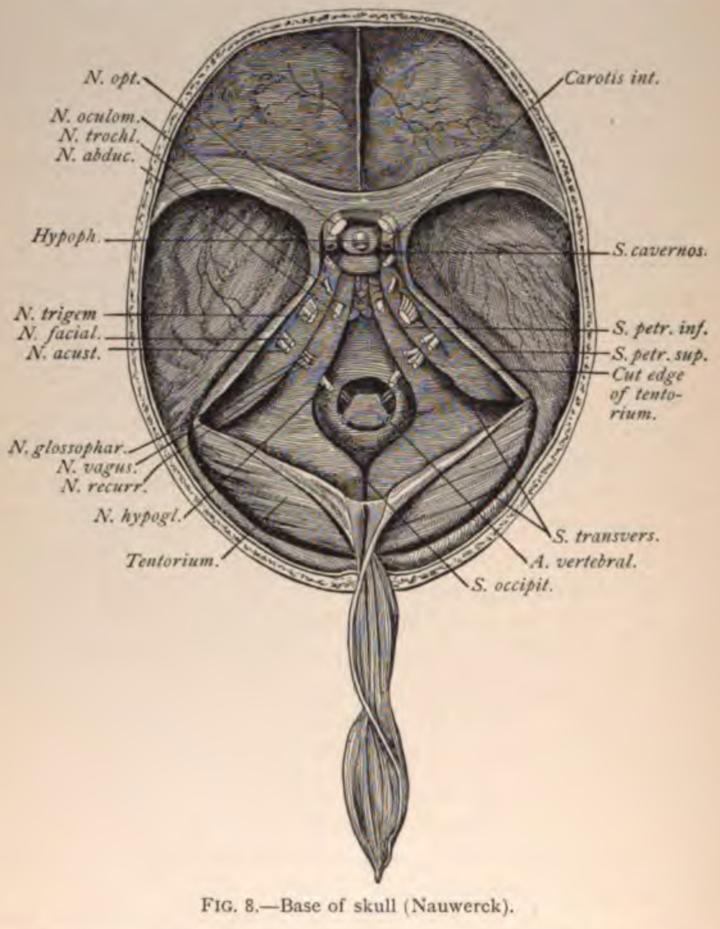

FIG. 8.—Base of skull (Nauwerck).

in toward the foramen magnum. Then cut the nerves given off from the medulla oblongata while supporting the convexity of the brain in the left hand.

Lastly, carry the knife as far as possible into the spinal canal, and divide the cervical cord by an oblique incision from each side, severing the vertebral arteries with the same stroke. Better than a knife is the myelotome, because it gives a cross-section of the cord and allows more of it to be removed.

The brain is now to be removed by passing the first and second fingers of the right hand in on either side of the cord, and everting the brain while still supporting it posteriorly with the left hand.

Before proceeding to open the brain it is best to examine the base of the skull, particularly the dura, of which the sinuses should be incised, and the hypophysis cerebri.

If there is a suspicion of a fracture at the base, strip off the dura, so as to give a better opportunity for examination of the bone.

The brain should be weighed before it is dissected. The average weight in an adult male is 1358 grams; in an adult woman, 1235 grams.

External Examination of the Brain.—Place the brain with the base uppermost and with the cerebellum toward the operator. Examine first the pia and the cranial nerves, then the arteries, especially the middle cerebral and its branches on each side in the fissure of Sylvius, for it is here that emboli most frequently lodge. The pia bridging the fissure of Sylvius can sometimes be torn through, but usually has to be cut.

It is important, particularly in cases of obscure cerebral symptoms, to feel gently with the finger-tips all over the surface of the brain for any areas of increased density, because patches of sclerosis may in that way be found which might otherwise be overlooked.

By stripping off the pia—a procedure not often advisable—adhesions over pathological areas can sometimes be found pointing to the lesions beneath, but the pia should not be stripped from those portions which are to be examined microscopically. To remove the pia an incision is made on the median surface of each hemisphere just above the corpus callosum from one extremity to the other, and the pia stripped back first from the median and then from the convex surface. The stripping is done by means of the fingers, with occasional aid from the forceps.

Section of the Brain.—There are several methods of cutting up the brain, no one of which is particularly suitable to all occasions. That method must be chosen which is most fitted to the individual case and to the use to which the tissue is to be put.

The ideal method from a neuro-pathological standpoint would undoubtedly be to harden the brain entire, and then to make serial frontal sections thin enough for microscopical purposes through the whole organ. The nearest approach to this ideal method is to harden the brain entire in formaldehyde, a process occupying ten days to two weeks (see page 309), to make thin serial sections, to mordant the sections, divided if necessary into smaller pieces, in a chrome salt (preferably by Weigert's quick method), and then to carry through a number of series from the important parts for microscopical examination. By this means the relations of the various cerebral structures and of the pathological lesions can be perfectly preserved and studied. This method can be particularly recommended for tracing degenerations in the motor tract.

If there is a noticeable focal lesion, such as a tumor or hemorrhage, it should be so incised, generally frontally or horizontally, as best to show its relations to the important cerebral tracts and ganglia. In these cases also the best results are obtained by hardening the brain entire in formaldehyde, and later making serial sections for macroscopic study or for carrying through for histological purposes. In many cases, however, it is necessary or advisable to examine the lesions in the fresh state. For instance, if it be desired to study the neuroglia-fibers, it is positively necessary to cut out thin slices of fresh tissue and to fix them immediately in the proper solution. Often, too, the lesion cannot be or is not found except on fresh examination, or the clinician whose case it is desires to see at once the cause of certain symptoms. Under such circumstances the more ideal method must be sacrificed, and as much made out of the case as is possible in the condition in which it is left after the examination.

For the routine examination of the brain, to demonstrate its topography and to bring to light suspected or unsuspected lesions, probably no method is more generally used than Virchow's. The objection most often made against it is that the cerebral cortex is too much cut up. In case, however, it is desired to preserve the cortex or parts of it for microscopic purposes, the longitudinal incisions after the first may be omitted, and the cortical portion, after being separated from the stem, may be cut in any way that seems advisable. In like manner, the brain-stem or any other part

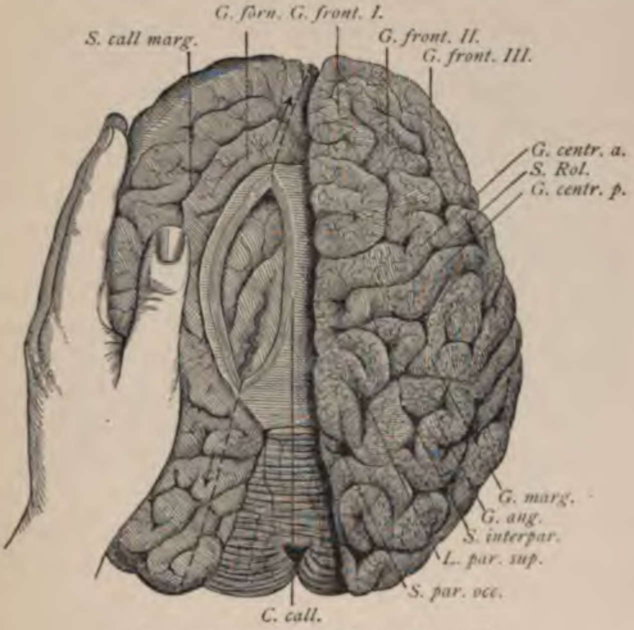

FIG. 9.—First cut in the brain (Nauwerck).

may be left uncut, and hardened entire in formaldehyde for histological purposes.

Virchow's Method.—The brain is to be placed on its base in the same position as one's own. Press the hemispheres apart a little so as to expose the corpus callosum. Hold the left half of the cerebrum in the left hand with the fingers on the lateral aspect and the thumb in the longitudinal fissure. Then make an almost vertical incision with a long, slender knife through the roof of the left ventricle in its middle third, 2 to 3 mm. from the median raphé of the corpus callosum. The roof of the ventricle is to be slightly raised vertically by the thumb, so that the incision, which must not be too deep, may not injure the basal ganglia. The incision is to be continued into the anterior and posterior cornua. Then make a long incision from one end of the above cut to the other, passing just outside of the basal ganglia at an angle of about 45°. Repeat the process on the right side, turning the brain half around. Next seize what remains of the corpus callosum and fornix in the middle, lift them, and cut through from below up, passing the knife through the foramen of Munroe. The parts are then turned back, exposing the velum interpositum and the choroid plexuses. By drawing back the velum interpositum the third ventricle is uncovered.

The corpora quadrigemina are exposed by cutting transversely the right posterior pillar of the fornix and adjoining brain-substance and carrying them over to the left. Each ventricle as it is opened is to be carefully inspected and any abnormal condition of its ependyma noted. The cortex is further divided on one side, and then on the other, by holding it in the left hand and making vertical straight sections from the upper angle of the previous cut into the convex cortex, allowing the sections to fall apart, so as to avoid touching and soiling the surface with knife or fingers. Each portion thus cut represents a prism. The incisions should go well into the cortex, but not so far as to separate the different pieces. The basal ganglia are examined by means of a number of frontal sections. For this purpose the left hand is placed palm upward underneath the brain, so that as each section is made over the tips of the fingers by one long stroke of the knife it falls forward, exposing a clean surface of which the two halves can be compared. An incision is next carried through the middle of the pineal gland, the corpora quadrigemina, and the vermiform process of the cerebellum, opening the aqueduct of Sylvius and the fourth ventricle.

Each half of the cerebellum is divided by a median horizontal section into halves, and these portions are still further subdivided by a series of cuts radiating from the peduncles.

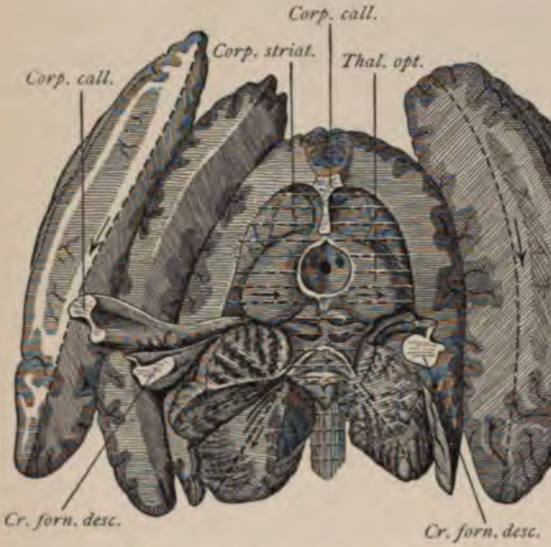

FIG. 10.—Section of the brain (Nauwerck).

In order to make sections of the pons and medulla the brain is folded together and turned over. Several cross-sections are then made with the left hand placed beneath as in sectioning the basal ganglia.

Before making the sections it is well to remove the basilar and vertebral arteries, especially if they are calcified.

In *Pitre's method* of dissecting the brain the lateral ventricles are opened as in Virchow's method. Then the pedunculi cerebri are cut squarely across, so as to remove the pons and cerebellum, and a longitudinal incision is carried down through the third ventricle, halving the cerebrum. Through each half of the cerebrum a series of six sections is then made parallel to the fissure of Rolando. The names of the sections and the important parts which they show are as follows:

1. The *pre-frontal section* through the frontal lobe, 5 cm. anterior to the fissure of Rolando, shows the gray and white substance of the frontal convolutions.

2. The *pediculo-frontal section* through the posterior portions of the three frontal convolutions shows the anterior extremity of the island of Reil, the lenticular and caudate nuclei, and the internal capsule.

3. The *frontal section* through the ascending frontal convolution, parallel to the fissure of Rolando, shows the optic thalamus, the lenticular and caudate nuclei, the claustrum, the external and internal capsules, the anterior portion of the descending horn of the lateral ventricle, and the island of Reil.

4. The *parietal section* through the ascending parietal convolution shows portions of the same structures as the preceding, and a transverse view of the hippocampus.

5. The *pediculo-parietal section* through the parietal lobe, 3 cm. posterior to the fissure of Rolando, shows the tail of the caudate nucleus in two places and the posterior portion of the optic thalamus.

6. The *occipital section* through the occipital lobe, 1 cm. in front of the parieto-occipital sulcus, shows simply the white and gray matter of the occipital lobe. The cerebellum, pons, and medulla are incised in the manner already described.

Removal of the Spinal Cord.—The body is to be placed face downward, with the head over the end of the table and a block under the chest. The incision is made over the spinous processes from the occiput to the sacrum. Dissect the skin and muscles back on each side, so as to leave the vertebral laminæ as bare as possible. The laminæ may be cut through by means of several instruments, of which the double-bladed saw (Luer's rhachiotome) is perhaps the safest, at least for beginners. The single-bladed saw with rounded end is also very useful and can be thoroughly recommended. The operation can be done most quickly by biting off the spinous processes with the heavy bone-forceps and cutting through the laminæ with chisel and hammer, but there is greater danger of injuring the cord.

The numerous artifacts in the cord, reported as neuromata and heteroplasia even within very recent times by competent pathologists, would seem to indicate that the need of careful and delicate technique in the removal of the spinal cord is not yet fully appreciated.

The laminæ should be sawn nearly or entirely through in a line with the roots of the transverse processes from the third or fourth lumbar vertebra to the cervical region. The arches of the cervical vertebræ are best divided with a heavy bone-cutter, because they cannot be easily sawn, and there is sufficient room here for the points of the bone-cutter without danger of their pressing on the cord.

It is important to strike the outside limits of the spinal canal, so as to get as much room as possible for the removal of the cord. Test if the sawing be deep enough by the mobility of the spinous processes. If necessary, they can be freed by means of the hatchet-chisel and a hammer in the same way that the calvaria is loosened.

As the cord reaches only to the second lumbar vertebra, cut through between the third and fourth, free with the heavy bone-cutter the lower end of the row of the spinous processes, which are held together by their ligaments, and strip them up to the neck; then cut through the cervical arches with the bone-cutter, taking care that the point within the canal does not come in contact with the cord.

The nerve-roots are to be divided with a sharp scalpel by means of a long cut on each side of the cord. Then cut across the dura and the nerve-roots at the lower end of the exposed canal, and, while holding the dura with forceps, carefully free the cord from below up with scissors or scalpel, taking care all the time not to pull or bend the cord, because in either way artifacts may be produced. Cut the cord squarely across as high in the cervical canal as possible, so that the remaining portion may be easily removed with the brain.

Lay the the cord after removal on a flat surface and incise the dura longitudinally, first posteriorly and then in front. A series of cross-sections, usually 1 to 2 cm. apart, is made through the cord while supported on the fingers during the cutting, so that the cut surfaces shall fall apart. The different segments should ordinarily be left attached to the dura, so that their position in the cord can easily be determined.

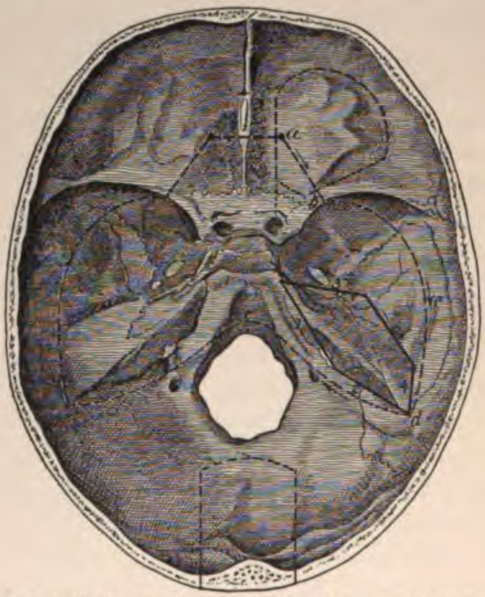

FIG. 11.—Base of skull, showing lines of incision for removing internal eye, etc. (Nauwerck).

A diagnosis from the fresh, macroscopic appearances of the cord is often very difficult to make, according to the best authorities.

The Eye.—The contents of the orbit, including the posterior part of the eye, can be readily examined by chiselling off the roof of the orbit. The posterior half of the eye can be removed by cutting around the eyeball with sharp scissors without changing the hold of the forceps on the sclera. If done quickly, the retina remains quite well spread out. The anterior half of the eyeball is to be propped in place by a plug of cotton dipped in ink or in a solution of permanganate of potassium.

The Ear.—The middle ear can be exposed by chipping off with a chisel its roof, which lies in the middle of the petrous portion of the temporal bone. The roof can also be very easily bitten off with the heavy bone-cutters. If, however, it be desired to examine the ear more carefully by means of a section through the external meatus and the middle ear, it will be necessary to remove the whole of the petrous bone. For this purpose the incision behind the ear must be carried back along the anterior edge of the trapezius muscle halfway down the neck. Then the skin-flaps, including the external ear and the underlying tissues, must be dissected back for some distance on each side of the incision. Two converging incisions are then to be sawn, the anterior passing through the root of the zygomatic arch, the posterior just back of the sigmoid sinus, so as to come together at the apex of the pyramid of the petrous bone, or, better still, to meet in the foramen magnum. An ordinary chisel and a hammer or mallet will be found very convenient for freeing the petrous bone after the incisions have been sawn.

In the examination of the petrous bone after it has been removed the first step is to chisel off the tegmen tympani so as to get a view of the middle ear. Next remove the lower wall of the external meatus, so as to expose the outer surface of the membrana tympani. Finally divide the petrous bone with a fine hair-saw by an incision starting in at the styloid process and coming out at the carotid canal, parallel to the crest of the pyramid of the petrous bone.

This incision divides the cavum tympani into halves. In the lateral half can be seen the membrana tympani with the hammer and the anterior half of the mastoid cells. In the median half are the labyrinthine wall of the cavum tympani with the stapes and the posterior half of the mastoid cells. It is best to remove the anvil before sawing through the bone. The Eustachian tube can be easily exposed by starting from its termination in the middle ear.

The Naso-pharynx.—Although a fair view of the nares and pharynx can be obtained by chiselling off the portion of the base of the skull lying over them, the method does not begin to offer the satisfactory view that can be obtained by the method of Harke,[1] a method which is not so difficult as might at first sight seem, and which consists in halving the base of the skull by a longitudinal incision. To do this the original incision in the scalp must be extended on each side over the mastoid processes and along the anterior edge of the trapezius muscle to a point below the middle of the neck. Then the posterior flap and the underlying muscles must be freed from the occipital bone and the upper portion of the cervical vertebræ. In like manner, the anterior flap must be dissected from over the root of the nose and the upper edge of the orbits, and be drawn down over the face. Then flex the head strongly forward and saw through the occipital bone and the base of the skull, dividing the occipital and frontal bones, the sella turcica, the cribriform plate, and the basilar process into equal halves. Anteriorly, it is well to go a little to the left or right, so as not to injure the nasal septum.

The next step is to cut the pachymeninx and the apparatus ligamentosis between the anterior edge of the foramen occipitale magnum and the processus odontoideus, as well as the inner side of the atlanto-occipital joint from within. Then the two halves of the skull are to be drawn forcibly apart. The nasal bones, the hard palate, and the alveolar process of the upper jaw break, and the two halves of the base of the skull open like a book, revolving around an axis which passes through the joint of the lower jaw and the atlanto-occipital joint.

If the foramen occipitale magnum offer too much resistance, break through it with a chisel, and also if necessary through the anterior and posterior arches of the atlas.

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[1] *Berliner klin. Wochenschrift*, 1892, No. 30.

It is now easily possible to inspect the sinus sphenoidales, the nasal septum, the frontal sinuses, and the nasal passages. The antrum of Highmore is easily opened with forceps and a pair of bone-shears.

After the operation the two halves of the base of the skull are brought together, and wired if necessary. When the skin-flaps have been replaced all evidence of the operation is covered up.

Examination of New-born and Very Young Children.—1. The head is preferably opened by the method given on page 52.

2. According to Nauwerck, the spinal canal can be opened by dividing the vertebral arches with strong scissors.

3. The umbilical cord, if present, and the umbilical arteries demand close attention in children who have lived a few days or weeks, for the purpose of determining if infection has taken place at that point. Nauwerck advises a modification of the primary long incision. A little above the umbilicus it should divide into two diverging incisions running to the pubes. In this way a triangular flap is left containing the umbilical arteries, while from the upper end is given off the umbilical vein. The vessels may be ligated or opened at any point that seems advisable.

4. Anomalies of circulation should be looked for in all "blue babies." The closure or non-closure of the *ductus Botalli* (arteriosus) is best determined *in situ* by dissecting off the thymus and opening up the pulmonary vein in the middle of its anterior surface. The cut may be extended downward, if desired, through the pulmonary valve and the wall of the right ventricle. The duct lies in the median line of the pulmonary artery, a little above its division into its two main branches. A small probe can be passed through it into the aorta. The condition,of the *foramen ovale* between the auricles is easily examined.

For other anomalies of the circulation it will usually be found most satisfactory to remove the thoracic organs in mass, so as to be able to open up the heart and the vessels given off from it before any of the vessels have been severed from their connections.

5. In medico-legal cases especially it is important to determine whether or not a child has breathed. The main steps of the process are as follows:

(*a*) Position of the diaphragm before the chest is opened. When the lungs are fully distended it is at the fifth or sixth rib on the right and at the sixth rib on the left. When the lungs contain no air or are but partially distended the diaphragm reaches to the fourth rib.

(*b*) Ligate the trachea above the sternum before opening the thorax.

(*c*) After examining the heart, etc., divide the trachea above the ligature and remove the thoracic organs in one piece.

(*d*) Dissect off the thymus gland and the heart, and place the lungs in a large dish of clear cold water to see if they will float or not.

(*e*) Incise the lungs and notice if they crepitate; squeeze the lung-tissue gently, and see if bubbles of air mingle with the blood on the surface, or squeeze the lung beneath water and observe if bubbles of air rise to the surface. Decomposition may give rise to gas in the lungs.

(*f*) Divide the lungs into lobes, and then into small pieces, and determine if any of them will float.

Table of the Weight and Length of the Fetus at each Month of Gestation (from v. Hecker, cited by Nauwerck).

Time in months.	Weight.	Length.
2	4 gr.	2.5–3 cm.
3	5–20 "	7–9 "
4	120 "	10–17 "
5	284 "	18–27 "
6	434 "	28–34 "
7	1218 "	35–38 "
8	1549 "	39–41 "
9	1971 "	42–44 "
10	2334 "	45–47 "

6. The long bones should be incised, so as to expose the

epiphyseal line, which should be examined for evidences of congenital syphilis. The ends of the femur and tibia at the knee are usually chosen. For making the incision a fine hair-saw is preferable to a knife, because the latter often causes the bone to break apart at the epiphyseal line.

The age of the fetus in months can be determined after the fifth month by dividing the length in cm. by 5.

Weight of Organs in a New-born Child.

Brain	380 gr.	(Bischoff).
Thymus	14 "	(Friedleben).
Heart	20.6 "	(Thoma).
Lungs	58 "	
Spleen	11.1 "	
Kidneys together	23.6 "	(Thoma).
Testicles	.8 "	
Liver	118 "	

Restitution of the Body.—After an autopsy is finished it is necessary to put the body into such a condition that no evidence of the operation will be noticed except on careful inspection. All fluids should be removed from the cavities. Organs not required for further examination should be replaced. The brain is placed in the body-cavity because it is usually impossible to restore it to the skull. The best material for filling up the cavities is fine sawdust. It packs easily and smoothly, absorbs well, keeps the needle dry so that it does not slip, and does not interfere with sewing like oakum, which gets into the stitches. In private autopsies any makeshift, such as bran, newspapers, or cloth, must be employed. If the pelvic organs have been removed, stuff the pelvis tightly to prevent leakage. The cranium may be left empty, although it is usually better to pack a little sawdust or other material into the base of the skull and the upper part of the spinal canal to prevent leaking. Sometimes it is advisable to fill the cranial cavity with sand or sawdust wrapped tightly in a cloth, of which the edges are brought together and twisted so as to crowd the material into a compact mass. If the thoracic cavity is well packed with sawdust, the sternum will stay perfectly in place without being sewed.

If part of the vertebral column has been removed, a stick or heavy iron rod should be run into the spinal canal above and below, so as to stiffen the body and hold it in position while it is filled about half full of plaster of Paris. After this has set there is little danger of the body losing its form.

In sewing up the body-cavity, begin at the neck. Use a piece of twine a little over one and a half times the length of the incision. Take one stitch and fasten the end with a simple knot or with a surgeon's knot. Turn the loose end in under the skin. Hold the attached end of the twine taut with the left hand about 8 to 10 cm. from the line of incision. The needle is then passed from within outward through the edge of the flap and in a diagonal line from below upward. The stitches should be from 1 to 2 cm. apart, and about the same distance from the edge of the flap. The object of keeping the end of the twine taut is to keep the sutures tight and the edges of the flaps up so that the needle can be thrust in easily.

Arrived at the lower end of the incision, take two buttonhole stitches and draw them tight. Then take a long stitch off to one side and cut the twine close to the skin, so as to bury the end of it deeply and securely.

If in removing the calvarium the precaution is taken to crack at least a part of the inner table with the chisel and hammer, projecting pieces of bone are usually left which interlock and hold the calvarium snugly in position when it is replaced. It is further fastened by sutures on each side through the fascia of the temporal muscle. It is always more difficult to sew up the incision in the scalp than the one in the body, especially when the hair is long. Care should be taken to bury the ends of the suture securely.

The skull of a child is so thin that it is usually best to wire the calvarium in place or fasten it by means of double tacks, otherwise it may slip out of place after the scalp has been sewed up.

Slee's ingenious method deserves mention. The usual saw-cuts in the skull over the ear are allowed to cross each other, so that slits about an inch long are formed in the temporal bone. An ordinary roller bandage is stretched across the skull and crowded edgewise into the slits. Then the calvaria is replaced and the ends of the bandage are tightly overlapped over the vertex and secured by pins.

PART II.

BACTERIOLOGICAL METHODS.

I. CULTURE-MEDIA.

CULTURE-MEDIA consist of various nutritive substances, either liquid or solid, in or upon which bacteria will grow and multiply, and are, as a rule, contained in test-tubes ready for use.

The nutritive material in these test-tubes must be free from living bacteria—*i. e.* "sterile"—and must be kept so until used. This is accomplished by inserting a stopper of raw cotton into the mouth of each test-tube to exclude the entrance of bacteria from without, and then subjecting the tubes and their contents to the sterilizing action of live steam for the purpose of killing any bacteria which may have gained access to the medium during its preparation.

The Preparation of Test-tubes.[1]—*New test-tubes* should be washed in a very dilute solution of nitric acid (2–5 c.c. of the commercial nitric acid to the liter of water), then thoroughly rinsed in water and allowed to drain until dry or nearly so. The object of the use of the nitric acid is to remove any free alkali which may be present in the new tubes.

Old test-tubes containing culture-media, after removal of the cotton stoppers, should be boiled for from half an hour to one hour in a solution of common soda (4–6 per cent.). This treatment not only destroys bacteria, but it also loosens and liquefies the material in the tubes, so that it may be easily removed with the aid of a test-tube brush and plenty of water.

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[1] Test-tubes of the size known as 6 × ¾ in. are recommended.

When all the material has been removed from the test-tubes in this way, they are to be rinsed in clean water, then in the dilute nitric acid of the strength above indicated for the new test-tubes, and finally again rinsed in clean water, after which they are to be allowed to drain until dry or nearly so.

The test-tubes thus prepared are next to be provided with stoppers of raw cotton (not absorbent cotton), which are to be inserted into the mouths of the tubes for a distance of about 3 cm., and should fit the walls of the tubes smoothly. The stoppers should not be packed in nor fit too tightly, but be just firm enough in position to easily sustain the weight of the tube when it is lifted by the projecting portion of the cotton.

The stoppered tubes are then to be packed into a square wire basket which fits into the hot-air sterilizer,[1] and heated in this, with the door closed, until the temperature reaches about 150° C. The object of this heating is not to sterilize the tubes and cotton stoppers, but to mould the stoppers to the shape of the test-tubes, so that they can readily be replaced when removed in the subsequent filling of the tube with nutritive material. In packing the tubes into the square wire basket as many as possible should be placed with the cotton stopper uppermost, and the remainder of the space in the basket above the tubes may be filled with tubes placed on their sides.

PREPARATION OF CULTURE-MEDIA.

Bouillon.—Formula for 1000 c.c.:

Lean beef,	500 grams;
Or extract of beef,	3 "
Pepton,	10 "
Sodium chlorid,	5 "
Water,	1000 c.c.

500 grams, or about 1¼ pounds, of lean beef, finely minced, are thoroughly mixed with 1000 c.c. of ordinary tap-water

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[1] See any dealer's catalogue of bacteriological apparatus.

and the mixture is then boiled in a saucepan over the gas stove[1] for about half an hour. It is next filtered through filter-paper to obtain the clear infusion of the beef, free from the coagulated albumin and shreds of tissue. This clear beef-infusion is then turned back into the saucepan, which should be clean, and to it are added 10 grams of pepton (Witte), 5 grams of sodium chlorid, and sufficient water to make the total volume of the mixture 1000 c.c. The volume of 1000 c.c. may be indicated with sufficient accuracy by a mark previously made on the inner surface of the side of the saucepan. The mixture is next to be boiled until all these substances are dissolved, stirring frequently with a glass rod, and is then to be neutralized, for it has a decidedly acid reaction from the acid of the meat.

The neutralization is important and requires care (see also p. 83). The reaction required is that of a very faint alkalinity, as is shown by the production of a blue color on red litmus paper, while no change is produced on the blue litmus paper. In neutralizing, a 10 per cent. solution of caustic soda is added, a few c.c. at a time at first, and later, two or three drops at a time, while the mixture is kept boiling, the reaction being tested between each addition of alkali after thorough stirring with a glass rod.

The test of the reaction is best made by placing a drop of the mixture on a piece of litmus-paper by means of the glass rod and then moistening the paper at the water-faucet. In this way the best judgment can be formed of changes in the color of the paper. If the mixture becomes too alkaline, dilute hydrochloric acid is to be added to correct this.

When the proper reaction has been obtained the mixture is to be filtered through filter-paper into a flask, and sufficient water added to bring the volume of the filtrate up to 1000 c.c., thus replacing the loss by evaporation. The filtrate in the flask is now *bouillon*. If the bouillon be heated to the boiling-point, it will usually become more or less clouded by a precipitate of phosphates. As a rule, subsequent heatings do not cause any further precipitations. Therefore it is advisable, if it is desired to obtain perfectly clear bouillon, to steam the flask containing the freshly prepared bouillon in the steam sterilizer for about half an hour, and then, if the bouillon be clouded, to again filter, so that the subsequent sterilizations in the test-tubes will not cause precipitates.

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[1] In the preparation of culture-media some form of gas stove is preferable to a Bunsen burner.

The finely minced beef may be obtained in the shops under the name of Hamburg steak, or it may be very readily prepared with the aid of a meat-grinder.

The usual directions for the preparation of bouillon require that the mixture of the minced meat and water be allowed to stand over night in a cool place before boiling. In our experience this is not necessary.

For bouillon cultures the bouillon is run into test-tubes, each tube being filled to a depth of about 4 cm., and sterilized immediately and on the two following days, according to the general directions given on page 87, after which it is ready for use.

Bouillon may also be made as above indicated by using three grams of Liebig's extract of beef to the liter, instead of the beef-infusion. N.B.

Glucose Bouillon.—Formula:

Glucose (dry),	10 grams;
Lean beef,	500 "
Or extract of beef,	3 "
Pepton,	10 "
Sodium chlorid,	5 "
Water,	1000 c.c.

This medium is identical with the preceding, except that it contains 10 grams of glucose to the liter (1 per cent.) in addition to the other ingredients. The preparation of glucose bouillon is the same as that of plain bouillon, the glucose being added with the pepton and sodium chlorid.

Agar-agar (**plain**).—Formula for 1000 c.c.:

Agar-agar,	15 grams;
Lean beef,	500 "
Or extract of beef,	3 "
Pepton,	10 "
Sodium chlorid,	5 "
Water,	1000 c.c.

Agar-agar is essentially bouillon in which agar-agar has been dissolved so that a transparent jelly is formed. The function of the agar-agar is merely to give the medium the property of becoming liquid when heated and solid when cool; it is not nutritive. The nutritive substances are in the bouillon.

To make one liter, 15 grams of agar-agar are placed in the clear beef-infusion, made as described on p. 71 and boiled for one hour in a saucepan.[1] The agar-agar dissolves slowly, and continuous boiling is necessary to ensure its subsequent filtration. Before boiling, about 200 c.c. of water should be added to compensate for evaporation, and later, as the level of the liquid falls, more water should be added from time to time. It is well to have some mark on the side of the saucepan which will indicate the level of a liter. When the boiling is nearly finished, 10 grams of pepton, 5 grams of sodium chlorid, and sufficient water to make a volume of one liter are added to the mixture. The mixture is then neutralized, as described for bouillon, while still boiling.

After the boiling is completed the saucepan is to be placed in cold water until the temperature of its contents falls to about 60° C., as shown by the thermometer, the cooling being facilitated by stirring with a glass rod. When this temperature is reached, an egg is beaten into the mass and the saucepan with its contents replaced on the stove, where it is slowly brought to boiling and boiled for about ten minutes. The object of the adding of the egg is to clarify the medium. It is then filtered, boiling hot, through wet folded filter-paper into a flask. A funnel with corrugations on its sides is best to use. With this the folding of the filter-paper is not necessary.

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[1] If an autoclave (see p. 88) be available, it may be used very conveniently in hastening the solution of agar-agar in the meat-infusion. For this purpose the mixture of finely fragmented agar-agar and the beef-infusion should be placed in a Florence flask. When the temperature of the interior of the autoclave has reached about 120° C. or when the gauge shows a pressure of two atmospheres, the heat should be turned off and the apparatus allowed to cool to about 100° C. before opening. The mixture is then transferred to a saucepan and the preparation proceeded with as above indicated.

In order to save time, it is best to use two filters and two flasks at once, for the filtration rapidly becomes slow as the mass cools, and several heatings of the residue on the filter are necessary. As soon as the filtrate begins to appear slowly, drop by drop, the mass remaining on the filter should be turned back into the saucepan—which can best be done by making a hole in the bottom of the filter with the glass rod—and brought again to boiling. While boiling hot it is again poured on a fresh filter. This preparation of fresh filters and reheating may have to be repeated several times before all of the mixture is filtered. The filtration may also be carried on in the steam sterilizer to prevent the cooling of the medium.

When the amount of coagulated egg-albumin and medium remaining on the filter does not exceed a volume of 50–100 c.c., the filtration may be considered complete. To the filtrate, which is now agar-agar, is next added sufficient water to make up the loss by evaporation, and the medium is then to be run into test-tubes and sterilized, as described on page 87.

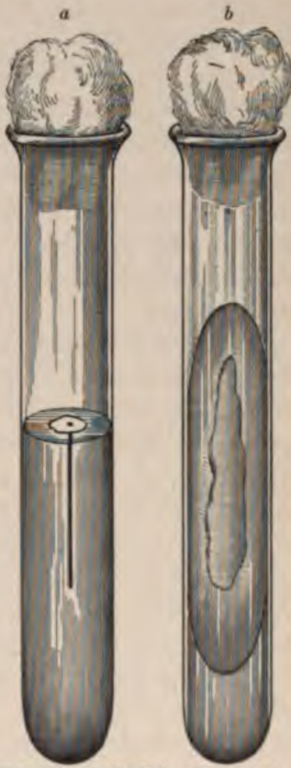

FIG. 12.—"Stab" culture (*a*); "slant" culture (*b*).

In view of the difficulty of filtering agar-agar, it has been proposed to avoid this operation by placing the fluid medium in a sedimenting vessel, such as a large funnel, with closed apex. The solid particles settle to the bottom if the medium be maintained in a fluid condition in the steam sterilizer for a certain length of time. When the medium has become solid it is turned out of the vessel as a cast, and the bottom portions, containing the sediment, cut off from it and rejected. The remaining portion will be found clear enough for most purposes and may be melted up at once for distribution in tubes, or if it now be desirable to further clarify it, it may be melted up and filtered as described above. It will be found to filter more readily than before.

Precipitates of phosphates in the medium frequently occur after the first sterilization, but if these be removed they do not usually appear again in subsequent heatings. Therefore, if it be desirable to obtain a very clear agar-agar, it is well to place the flask containing the freshly-prepared medium in the steam sterilizer for half an hour, and then filter again to remove any precipitate which may have appeared. The subsequent sterilization in the test-tubes will then cause no precipitation.

In filling the test-tubes it is customary to fill some tubes to a depth of about 3 cm. and others to a depth of about 5 cm. After the complete sterilization of the medium in the tubes as described on page 87, the first-mentioned tubes are placed on their sides with their mouths slightly elevated while the medium is still fluid, so as to form, after solidification, a slanting surface extending from near the bottom of one side of the tube to about half the length of the tube on the opposite side. The solidification of the agar-agar takes place in a short time, and as soon as it occurs the tubes are ready for use, this form of culture being known as a "slant" tube or culture (Fig. 12, *b*). It is well, however, to allow the tubes to remain in their slanting position for a day or two to permit the medium to become more or less adherent to the walls of the tube, and thus avoid its tendency to slide downward when the tubes are placed in the upright position.

The tubes filled to a depth of 5 cm. are to be allowed to cool and solidfy while in an upright position, and the form of culture-tube thus obtained is called a "stab" culture (Fig. 12, *a*), because the medium in the tube is inoculated for culture purposes by inserting an infected platinum wire into its depths.

Glucose Agar-agar.—Formula for 1000 c.c.:

Glucose (dry),	10 grams;
Agar-agar,	15 "
Lean beef,	500 "
Or extract of beef,	3 "
Pepton,	10 "
Sodium chlorid,	5 "
Water,	1000 c.c.

This medium differs from plain agar-agar only in the addition of 10 grams (1 per cent.) of glucose. The glucose should be obtained in the form of solid masses, not as a thick fluid, and it is to be added with the pepton and sodium chlorid. In short, glucose agar-agar is made with glucose bouillon in identically the same manner that plain agar-agar is made with plain bouillon.

Lactose-litmus agar-agar consists of plain agar-agar to which has been added 2 or 3 per cent. of lactose and sufficient litmus tincture to give it a pale-blue color.

Glycerin Agar-agar.—Formula for 1000 c.c.:

Glycerin, c. p.,	60 c.c.;
Agar-agar,	15 "
Lean beef,	500 grams;
Or extract of beef,	3 "
Pepton,	10 "
Sodium chlorid,	5 "
Water,	1000 c.c.

This medium is prepared by adding to plain agar-agar after its final filtration, and before running it into the test-tubes, 60 c.c. (6 per cent.) of glycerin c. p., and mixing thoroughly.

Gelatin (plain).—Formula for 1000 c.c.:

Gelatin,	100 grams;
Lean beef,	500 "
Or extract of beef,	3 "
Pepton,	10 "
Sodium chlorid,	5 "
Water,	1000 c.c.

Gelatin is essentially bouillon in which gelatin has been dissolved, so that a transparent jelly is produced which is solid at ordinary temperatures and fluid when slightly warmed. To prepare one liter, 100 grams (10 per cent.) of *golden seal French gelatin* are dissolved in a liter of the hot bouillon which has been heated to boiling in a saucepan. When the gelatin is thoroughly dissolved the mixture is boiled for about five minutes, and the marked acidity of the gelatin then carefully neutralized by the addition of caustic soda, in 10 per cent. solution, to a very faint alkalinity, as has been described in the preparation of bouillon. As in the case of agar-agar, the mass is then cooled to 60° C., an egg beaten into it, then gently heated again to boiling, and boiled about ten minutes, when it is to be filtered through a wet folded filter into a flask. Gelatin usually filters fairly rapidly, but time may be saved by using two filters at once. When filtered it is to be run into test-tubes and sterilized, as described on page 87. It is used both in the form of "slant" and "stab" cultures, as in the case of agar-agar (see page 76).

In the preparation of this medium it is important to subject it as little as possible to the boiling temperature, for prolonged exposure to this destroys its power of solidifying. Therefore in sterilizing, gelatin tubes should never be allowed to remain exposed to live steam longer than twenty minutes. It is also important to apply the heat slowly during the process of heating after the addition of the egg above mentioned, in order to avoid "burning."

Glucose Gelatin.—Formula for 1000 c.c.:

Glucose,	10 grams;
Gelatin,	100 "
Lean beef,	500 "
Or extract of beef,	3 "
Pepton,	10 "
Sodium chlorid,	5 "
Water,	1000 c.cm.

This medium is essentially gelatin dissolved in glucose bouillon (see page 73), and is prepared in the same manner as the plain gelatin, except that glucose bouillon is used instead of plain bouillon.

Blood-serum (Löffler's Mixture).—Formula:

Glucose bouillon (see page 73),	1 part;
Beef blood-serum,	3 parts.

This culture-medium consists of a mixture of the blood-serum of the bullock and glucose bouillon, which is run into test-tubes and coagulated by heat in such a way as to form a slanting surface for culture purposes—*i. e.* it is used in the form of "slants."

The *blood-serum* is collected at the slaughter-house in tall glass jars of the capacity of a gallon or more. These jars should be thoroughly clean, but sterilization is not necessary.

The blood which is obtained by the Jewish method of slaughter—viz. by severing the carotid artery—is the best for the purpose, because it clots more readily. As the blood runs from the vessels of the animal it is received in the glass jar, and immediately placed in a cool place for twenty-four to forty-eight hours to allow it to clot and the serum to separate. All unnecessary agitation of the fresh blood should be avoided, as this interferes with its proper clotting. It is well to inspect the blood after a few hours, and gently loosen with a clean glass rod any adhesions which the clot may have formed to the wall of the jar, thus allowing the clot to more readily contract and squeeze out the serum from its meshes. After about twenty-four hours the serum is removed by the aid of a clean pipette and brought to the laboratory. If the clot is in good condition, more serum will appear after another twenty-four hours, and if necessary this also may be used.

The presence of red blood-corpuscles in the serum is of little importance. *Three parts* of the beef blood-serum thus obtained are to be thoroughly mixed with *one part* of glucose bouillon (*vide supra*), convenient quantities being 900 c.c. of *blood-serum* and 300 c.c. of *glucose bouillon*.

This mixture is then run into test-tubes as described on page 85. The quantity run into each test-tube should be sufficient to fill it to a depth of about 3–4 cm. The tubes containing the requisite amount of the mixture are next subjected to the action of heat while in a slanting position, so that the mixture in the tubes may become solid or coagulated, and so offer a smooth slanting surface for culture purposes extending from a point near the bottom of the tube to about halfway up the opposite side or higher.

The coagulation is effected either in the hot-air sterilizer by packing the tubes on their sides, the proper slant being secured by means of strips of cardboard placed between the layers of tubes, or better, in the blood-serum coagulator which may be obtained from dealers in bacteriological apparatus.

If the hot-air sterilizer is employed, the temperature should not exceed 90° C. nor fall below 85° C., and the door should be kept closed. It is optional whether the sterilizer be packed full of tubes or only a few layers of tubes be coagulated at a time, with careful watching to avoid overheating. In the former case two or three hours will be required to firmly coagulate the tubes in the middle layers, while the lower layers may be overheated. To avoid this overheating of the lower layers, a false bottom or one or two layers of empty tubes may be employed.

The blood-serum coagulator is much more convenient and gives much more satisfactory results. The temperature of the interior should be kept at about 95° C. To save time in heating, the apparatus may be filled with hot water from the hot-water faucet.

Whichever apparatus is employed for coagulation, it is of the utmost importance that the coagulation of the mixture be a thorough one, and that the medium in the tubes becomes firm and solid, otherwise bubbles and cavities will form in it and destroy its smooth surface when it is subjected to the subsequent steam sterilization. When the tubes are firmly coagulated they are to be packed with the cotton stopper uppermost in a round wire basket and sterilized by steam three times, as indicated on page 87, after which they are ready for use.

This method of preparing blood-serum tubes is very different from the one usually described, a most tedious and time-consuming procedure, requiring a high degree of technical skill, by which it is practically impossible to make use of blood-serum tubes for ordinary purposes.

With the method here detailed we think that the best culture-medium for the routine examination of pathological material is obtained. It is preferred by us for various reasons, chief of which are as follows:

First, the ease and facility with which it can be prepared, especially when a proper coagulating apparatus is available.

Secondly, the greater and more rapid growth of certain important pathogenic bacteria upon it than upon ordinary media.

In the method usually described the serum (which should be clear or free from blood-corpuscles) is obtained under all aseptic precautions, is carefully mixed with sterile glucose bouillon in the proportions given above, and the mixture then run into sterile test-tubes. During all the manipulations precautions are necessary to avoid contamination, the serum never being allowed to come in contact with any object which is not sterile, and exposure to the air during the processes of transference from one vessel to another avoided as much as possible.

The mixture now being in test-tubes, it is subjected for one hour on each of five successive days to a temperature of 68° to 70° C. in a chamber provided with a water-jacket. This temperature is sufficient to kill the vegetative forms of any bacteria which may be in it, but does not coagulate the medium. The intervals between the sterilizations are for the purpose of allowing any spores to develop into the vegetative form and thus become susceptible to the destructive action of heat.

After the fifth sterilization the medium is solidified in the tubes in the form of "slants" by slowly raising the temperature of the chamber to about 80° C., and keeping the tubes at this temperature for several hours. In solidifying the great object is to obtain a gelatin-like, fairly transparent medium and to prevent opacity. To attain this it is necessary to proceed very carefully with the heating and avoid overheating or too rapid heating, the tubes being inspected from time to time and removed from the chamber as soon as their contents have the proper consistency. When gelatinized the tubes are placed in the incubator for twenty-four hours to determine whether they are sterile, after which they are ready for use.

The blood-serum medium produced by this older method is especially suited for the cultivation of certain pathogenic bacteria—for instance, the bacillus tuberculosis and the bacillus diphtheriæ—but we do not think that its superiority in this respect over the more readily prepared, firmly coagulated form above described is sufficiently marked to compensate for the great difficulties in its preparation.

Litmus-milk is a form of culture-medium used for determining certain of the physiological properties of bacteria. It consists of cow's milk which has been colored blue by litmus and containing a minimum amount of cream. A pint or so of strictly fresh milk is placed in a flask and steamed in the steam sterilizer for about half an hour. When it is removed it will be found that most of the cream has collected at the surface, and it is then easy to draw off the milk from the deeper layers with a pipette into a separate flask. To the milk from which most of the cream has been thus removed is added sufficient of an aqueous solution of litmus (freshly filtered) to give it a pale-blue color. The colored milk is then run into test-tubes (5 cm. deep in each tube) and sterilized, as indicated on page 87, after which it is ready for use. It is of great importance that the milk be fresh. If it is not, it may contain spore-bearing bacilli which it is practically impossible to kill by the steam sterilization.

Potato-cultures according to Bolton.—Potatoes—preferably old ones—are first washed to remove all the coarser particles of soil, and then solid cylinders are cut out of them with a cork-borer or apple-corer. These cylinders should be of a suitable diameter to fit into the test-tubes used for other culture-media, and should be about 5 cm. long. They are then cut longitudinally in an oblique direction with a sharp knife, so that a smooth slanting surface is produced, beginning near one end and extending diagonally to the other end. The pieces of potato thus prepared are next to be washed in running water over night. After washing, each piece is placed in a test-tube, the larger end resting on the bottom of the tube, a few drops of water being added to prevent drying, and then sterilized as indicated on page 87. If desired, a small piece of glass rod may be placed in the bottom of the tube to elevate the potato above the few drops of water (Fig. 13).

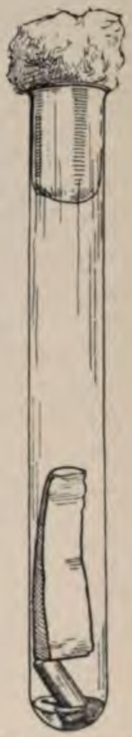

FIG. 13.—Potato-culture.

Dunham's Pepton Solution.—Formula for 1000 c.c.:

Pepton,	10 grams;
Sodium chlorid,	5 "
Distilled water,	1000 c.c.

The pepton and sodium chlorid are dissolved by boiling and the mixture filtered. The clear filtrate is then run into test-tubes, each test-tube being filled to a depth of 5 cm., and is to be sterilized as indicated on page 87, after which it is ready for use.

The Adjustment of the Reaction of Culture-media by Titration.—Because comparatively small variations in the reaction of culture-media may have a marked effect upon the morphology and mode of growth of bacteria grown upon them, a more exact adjustment of their reaction than is possible with litmus paper is desirable. This is especially important for media used for the cultivation of the bacteria of water, of soil, and of the air. For ordinary purposes of cultivation of bacteria, especially of the pathogenic forms, the adjustment of the reaction with litmus paper, as elsewhere described, if carefully done, will be found to be sufficient.

The more exact method of adjusting a reaction is one of titration with phenolphthalein as an indicator. The method is as follows: When the culture-medium, whether it be bouillon, agar-agar, or gelatin, has been neutralized with the aid of litmus paper, made up to the proper volume, and when it is all ready for filtering, as described elsewhere, 5 c.c. of it are transferred by means of a pipette to a 6-inch porcelain evaporating dish; to this 45 c.c. of distilled water are added, and the 50 c.c. of fluid are boiled for three minutes over a flame to expel any carbon dioxid which may be present.

Next, 1 c.c. of a 0.5 per cent. solution of phenolphthalein in 50 per cent. alcohol is added to the mixture in the dish, and immediately after this enough of a *twentieth normal* solution of sodium hydroxid is cautiously run into the dish, from a burette, to produce a pink color in the mixture. The judgment of the proper color which indicates that sufficient alkali has been run in requires some practice. The color to be obtained is a bright pink. The appearance of the proper pink color is preceded by a pinkish darkening of the fluid which may deceive the inexperienced.

The quantity of the twentieth normal sodium hydroxid solution required to effect this result is then read off from the burette. The number of cubic centimeters required denotes the percentage by volume of a *normal* solution of sodium hydroxid which would be required to make the total volume of culture-medium neutral to phenolphthalein. That this is so will be apparent after a simple calculation.

The reaction recommended by the Bacteriological Committee of the American Public Health Association as a standard to which culture-media should be adjusted is such that 1.5 per cent. of a *normal* solution of sodium hydroxid would be required to be added to the medium to make it neutral to phenolphthalein. This reaction corresponds closely to a faint alkalinity toward litmus, for the neutral point of phenolphthalein is not identical with that of litmus. The adjustment of the reaction to this standard is effected by adding to the bulk of the culture-medium sufficient normal sodium hydroxid solution or normal hydrochloric acid solution.

For example: If the titration shows that 5 c.c. of the medium requires 1.9 c.c. of the twentieth normal solution of sodium hydroxid to make it neutral to phenolphthalein, then the total mass of the medium will require the addition of 1.9 per cent., or 19 c.c. for a liter, of a normal solution of sodium hydroxid to make it neutral; but the reaction required is such that 1.5 per cent. of a normal solution of sodium hydroxid should be required to make it neutral. Therefore, 0.4 per cent, or 4 c.c. for a liter, of a normal solution of sodium hydroxid should be added to the main mass of the medium.

When the calculated amount of normal solution has been thoroughly mixed with the medium and the latter boiled for a few minutes, the titration should be repeated as above described. If the desired reaction is not found to be present, then further adjustment by addition of the calculated amount of normal acid or alkali solution should be made. It is not to be expected that the first addition to the medium of the calculated amount of normal solution will give exactly the required reaction in every case. This is due to unknown side reactions which take place in the culture-media.

When the reaction has been sufficiently adjusted, the medium is to be filtered and is then ready to be distributed in test-tubes.

The methods of making the normal and twentieth normal solutions required may be found in standard works on chemistry. If one has not some knowledge of chemistry, he would better have the solutions made by a chemist.

The filling of the test-tubes with the fluid culture-media described in this section is best effected by means of a funnel of a capacity of about a liter. In this the fluid medium is placed, and by means of a pinch-cock the requisite quantity of medium is run into each test-tube. In running the medium into the test-tubes the left hand holds the test-tube while the right hand removes the cotton stopper and manipulates the pinch-cock (Fig. 14). Care should be exercised not to allow any of the medium to come in contact with the neck of the test-tube, for it will make the cotton stopper stick to the walls of the tube. To avoid this, the delivery-tube of the apparatus should be inserted some distance into the test-tube in filling.

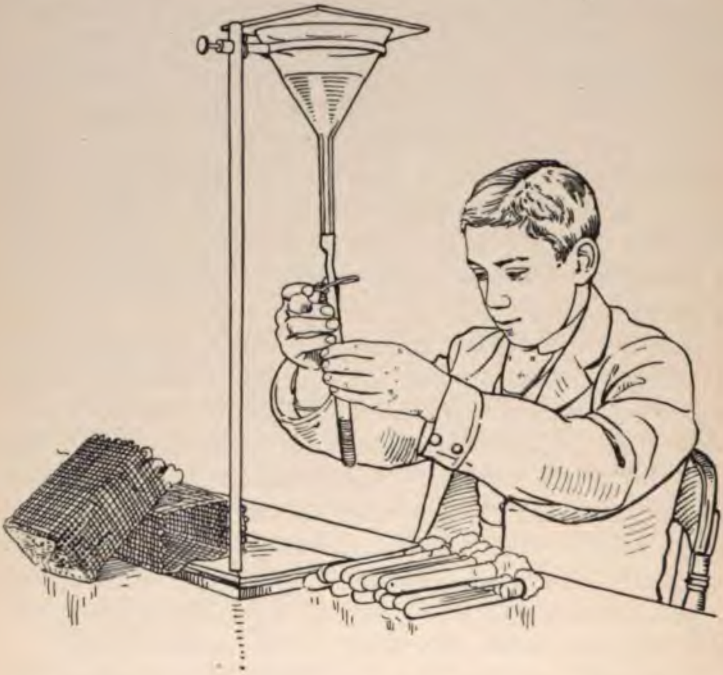

FIG. 14.—Method of filling test-tubes with culture-medium (Warren).

The *quantity* of culture-medium run into each test-tube varies according to the form of culture desired and the character of the medium. In the case of liquid media and solid media designed to be used in the form of "stab" cultures the tubes should be filled to a depth of 5 cm. For "slant" cultures of solid media a depth of about 3 cm. is sufficient, or enough to give a slanting surface from the bottom of the tube to about halfway up the opposite side.

Small *Ehrlenmeyer flasks* are sometimes used for bouillon cultures. These are of about 100 c.c. capacity, and are filled to a depth of about 1 cm. with the medium. The necks are provided with cotton stoppers, and the whole sterilized and treated as test-tube cultures.

STERILIZATION OF CULTURE-MEDIA.

In general, the sterilization of culture-media is effected by allowing them to remain exposed to the action of live steam in the steam sterilizer for twenty to forty-five minutes on three successive days. The period of exposure to live steam varies somewhat with the kind of culture-medium. A single exposure for the time mentioned is sufficient to destroy all bacteria present in what is called the vegetative or non-resistant form, but it will not kill *spores*, which represent a stage in the life-history of certain bacteria, in which form the organism is highly resistant to sterilizing agents.

Under favorable conditions, such as are to be found in culture-media at ordinary room-temperature, these spores develop into the vegetative or non-resistant form, which are easily destroyed by heat. Therefore, in order that the culture-medium be made sterile, it is necessary that it be again subjected to the action of steam on the following day for the same length of time, when the vegetative forms of the few surviving spores will have developed, and will be capable of destruction by ordinary exposure to live steam. As a further precaution a third similar sterilization on the next day is necessary. Therefore, three steam sterilizations, of from twenty minutes to one hour each, on successive days, are required to keep culture-media sterile for an indefinite period.[1]

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[1] As has been pointed out by Theobald Smith, this intermittent sterilization at 100° C. may not be sufficient in some cases to kill all the spores, because the condition in the media may not be favorable for their development into vegetative forms, between sterilizations. This seems to be especially true

A freshly prepared culture-medium must be sterilized on the same day that it is prepared, or by the next day it may be found to contain living bacteria, especially if kept over night in a warm room.

For the purpose of sterilization the test-tubes containing the media are to be placed in a round wire basket which fits into the steam sterilizer,[1] thus facilitating the handling of the tubes and also keeping them upright.

If the medium be in a flask ready for running into test-tubes, and if it be not convenient to do this the same day, the medium may be preserved as long as desired by inserting a cotton stopper into the mouth of the flask and then sterilizing as above indicated.

The time of each sterilization for bouillon, agar-agar, blood-serum, etc. may be fixed at half an hour; for potato-culture tubes and for litmus-milk, forty-five minutes.

In the case of gelatin, however, the time of exposure to live steam should be shorter, owing to the danger of destroying the solidifying power of the medium by too much heating. Twenty minutes' exposure is sufficient.

Large quantities of culture-media contained in flasks should be sterilized for forty-five minutes to an hour, for obvious reasons.

The sterilization of culture-media may also be effected in an **autoclave.** This is a steam-tight chamber for sterilizing by steam under pressure. Various forms of this apparatus are on the market. The great advantage of the use of this apparatus is that a single sterilization is sufficient. Exposure of culture-media in tubes, of glassware, and of other apparatus, in it to a temperature of 110° C. (6 lbs. pressure) for fifteen minutes suffices for sterilization in most cases. For the

of certain anaërobic spore-producing bacilli. Such spores may be the source of contamination of the culture-medium when it is placed under anaërobic conditions, or when the medium is used for anaërobic cultures, because strictly anaërobic bacteria may grow in company with other bacteria under aërobic conditions. Therefore, in those cases in which it is important to be certain of the absolute sterility of the culture-medium, sterilization in the autoclave (q. v.) is necessary.

[1] The "Arnold Steam Sterilizer" No. 5 is recommended.

sterilization of culture-media in bulk, about thirty minutes at this temperature is necessary.

In using the autoclave it is requisite that the confined air be replaced by superheated steam. To insure this, the time of sterilization should be reckoned only from the time when the theoretical temperature, as registered by the pressure-gauge, corresponds with that recorded by the thermometer.[1]

The Storage of Culture-media.—In order to prevent evaporation and the invasion of moulds, the cotton stopper should be cut off close to the mouth of the tube or flask, the surface of the stopper well singed with a flame, and the mouth of the tube or flask tightly closed with a cork.

Immediately before insertion, the portion of the cork that enters the tube or neck of the flask should be charred in a flame. If thought desirable, the cork may be sealed with paraffin.

II. BACTERIOLOGICAL EXAMINATIONS.

THE bacteriological examination of material obtained from the individual during life or at autopsies should determine whether bacteria are present or not, and if present their species and comparative number. At autopsies the examination should also determine the extent of the distribution of any infecting bacteria throughout the principal internal organs.

This is accomplished chiefly by means of two methods of examination—viz., the direct examination with the microscope of cover-glass preparations, and the results of cultures made from the tissues. Both of these methods should be employed together, but the culture method is perhaps the most important. A third but less frequent method is the inoculation of animals with pieces of tissue or material taken from the body.

Methods of Collecting Material.—In the bacteriological examination of pathological material obtained from the individual during life, it is of obvious importance that the material be protected from the invasion of bacteria from without, and that in its collection every object with which it comes in contact be free from living bacteria.

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[1] Bacteriological Committee Report, *Jour. Amer. Pub. Health Assoc.*, Jan., 1898.

To fulfil these requirements the material may be conveniently collected in any of the following ways:

1. It may be obtained directly from the individual by means of the sterilized platinum wire, and cover-glass preparations, cultures, and, if necessary, animal inoculations, made at once.

2. Since a very small quantity of the material usually suffices for the purposes of examination, it may often be very conveniently collected and brought to the laboratory on the so-called "swabs," where it can be subjected to the various manipulations at leisure.

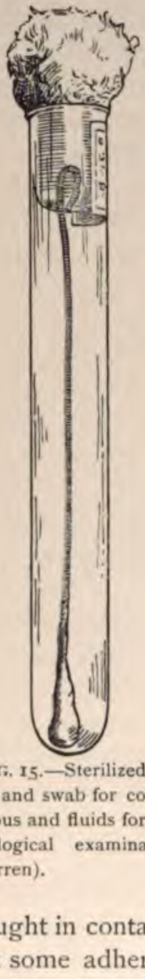

FIG. 15.—Sterilized test-tube and swab for collecting pus and fluids for bacteriological examinations (Warren).

The "swab" consists of a piece of rather stiff wire about six inches long, on one end of which is firmly twisted a pledget of absorbent cotton, so that the end of the wire is well covered. This is placed, cotton end first, in a test-tube, which is then provided with a cotton stopper (Fig. 15), and the whole sterilized in a hot-air sterilizer by heating to 150° to 180° C. during about half an hour. A large number of "swabs" in test-tubes may be kept on hand sterilized and ready for use.

When it is desired to secure material for bacteriological examination on a "swab," the cotton stopper is removed, the swab taken out, and the cotton end brought in contact with the pus or exudate in such a manner that some adheres to the cotton. The swab is then immediately replaced in the test-tube, the cotton stopper returned to its place, and the whole then carried to the laboratory.

In these manipulations care should be taken to avoid touching with the swab anything but the material which it is desired to examine, otherwise the material may be contaminated with other bacteria than those originally present in it.

By means of swabs material for examination from pus or exudates may be secured and brought to the laboratory in most instances. They are especially useful in surgical work, in which it is often desirable to determine the character of the organism present in a pus-formation or exudation without waiting to summon a bacteriologist or to collect the necessary cover-glasses, culture-tubes, platinum needle, etc. The swabs and their test-tubes may be kept on hand in a sterile condition, so that they may be handled by the operator or an assistant.

3. Fluid material may be collected by aspiration or otherwise. In the case of fluids care should be taken that everything with which the fluid comes in contact be clean and sterilized by heat if possible. The use of antiseptics, such as carbolic acid or corrosive sublimate, is to be avoided.

If a hypodermic syringe is used in obtaining material, it should be of a construction which will admit of sterilization by heat, and it should be so sterilized before using.

In the collection of pathological fluids, especially peritoneal exudates, a special form of apparatus has been found most useful. It consists essentially of a glass tube, about 14 cm. long and about 7 mm. in external diameter, one end of which is narrowed to a small opening and rounded off, while to the other end is attached a small rubber bulb like that on a "medicine dropper." It is to be kept ready for use in a test-tube, stoppered with cotton (see Fig. 16), the whole having been sterilized as are surgical dressings. The rubber bulbs are not expensive. Any number of pieces of this apparatus may be kept on hand in sterile condition.

When it is desired to obtain a sample of peritoneal or other fluid for bacteriological examination, the apparatus is removed from the test-tube and the fluid aspirated into it by manipulation of the rubber bulb. It is then replaced in the test-tube. The fluid thus obtained should be free from contamination and may be readily transported to the laboratory for examination.

Cover-glass Preparations.—The method of demonstrating the presence of bacteria in pathological material by means of cover-glass preparations depends upon the fact that bacteria have the property of being colored by certain of the aniline dyes, and thus may be more readily seen by the microscope. The cover-glasses are best kept in alcohol, and as required for use wiped dry with a soft cotton cloth. A cover-glass preparation is made as follows: A very small amount of tissue or material to be examined is thinly spread over the surface of a clean thin cover-glass with the platinum wire or "loop" described on page 96 so as to give a streaked appearance to the surface, but not a definite layer which is ordinarily too thick for satisfactory examination. The charged cover-glass is then dried by holding it in the fingers over the flame of a Bunsen burner, and when dry it is placed, charged surface uppermost, in the grasp of a pair of cover-glass forceps,[1] by means of which it is passed rapidly three times through the flame of a Bunsen burner or alcohol lamp. This "fixes" the material on the glass, and the preparation is then ready for staining by one of the various methods given below. In staining, the cover-glass is held by means of the forceps with the charged side uppermost and level, and the surface is then completely covered

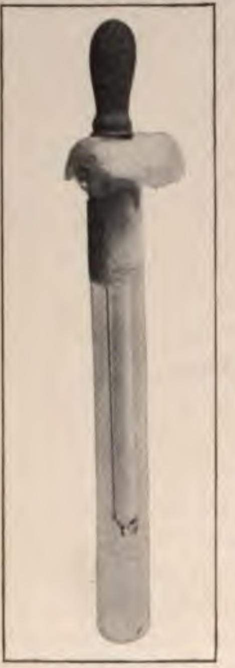

FIG. 16.—Apparatus for the collection of pathological fluids.

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[1] The form of cover-glass forceps known as Stewart's is recommended. They may be obtained from American dealers in bacteriological apparatus.

with the staining fluid, which is poured upon it from a dropping-bottle.[1] It may then be heated over the flame of the Bunsen burner, washed in water, and submitted to any further manipulation which may be necessary while still in the grasp of the forceps. When the staining is completed the preparation is next to be prepared for microscopic examination. This is done by placing the cover-glass, with as much water as will adhere to it, charged side downward, on a "slide," and then removing all remaining water, except a thin film of water between the slide and cover-glass, by gentle pressure with several thicknesses of filter-paper. The preparation is then ready for examination with an oil-immersion lens. The presence of this film of water is very essential for a satisfactory examination with the microscope, and its evaporation may be compensated for by a drop of water placed at the edge of the cover-glass. The preparation may also be mounted in balsam after carefully drying it first between filter-paper and then holding it in the fingers over the Bunsen flame, but the examination in water mount is better, because the apparent size of the bacteria is greater in this than when mounted in balsam.

It is of the greatest importance that cover-glass preparations be made from all exudates or acute inflammatory conditions of organs or tissues, so that the results of cultures can be controlled and some idea formed of the number and character of the bacteria present. They are of especial use as enabling one sometimes to recognize the presence of an organism which does not grow in the culture for some reason, and whose presence might escape notice, while their importance in many cases in the identification of the pneumococcus and the bacillus tuberculosis must be apparent. In certain cases it is best to examine the material in its natural state and without drying and staining. This is especially true of suspected infections with actinomyces and the amebæ coli. In these cases the material should be spread over a cover-glass, and this placed, while the material is still moist, charged surface downward, on a "slide," and then examined with various powers of the microscope. If necessary, a small amount of normal salt solution (0.6 per cent.) may be added to dilute the material and facilitate the examination.

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[1] The form of drop-bottle known by dealers in bacteriological supplies as the "T. K. patent," with flat stopper, is the best.

Staining Methods for Cover-glass Preparations.[1]—**Simple staining** is used for the demonstration of bacteria in general, and also useful in gaining an idea of the character of the cellular elements in the preparation.

Löffler's alkaline methylene-blue solution is perhaps the best staining fluid to use for simple staining, for it does not stain so diffusely and intensely as do the other commonly used dyes, such as fuchsin and gentian violet, which may also be employed.

The cover-glass, covered with the staining fluid, should be warmed over the Bunsen flame, so that the fluid steams, for about fifteen seconds. Boiling should be avoided. The preparation is then washed in water for two or three seconds and mounted.

Gram's Method of Staining.—1. Cover the preparation with aniline-gentian-violet solution for thirty seconds.

2. Wash in water for two or three seconds.

3. Cover the preparation with Gram's solution of iodin for thirty seconds.

4. Wash with 95 per cent. alcohol until the color ceases to come out of the preparation.

5. Wash in water for two or three seconds and mount.

Certain bacteria are stained by this method, while others are not. Bacteria when stained by it appear dark blue or black, while the nuclei of the cells are rather faintly stained or not stained at all. The method is especially useful in the demonstration of bacteria which are stained by it when they are present in small numbers or when a few Gram-staining bacteria are mixed among numbers of bacteria which do not stain by this method. It also has some value as a means of differentiating between bacteria which may be very much alike in size and shape.

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[1] The formulæ for staining fluids will be found in Part III. (see Index).

In the following table the behavior of the more important pathogenic bacteria toward the method of Gram is indicated:

STAINED BY GRAM'S METHOD.	DECOLORIZED BY GRAM'S METHOD.
Staphylococcus pyogenes aureus.	Gonococcus.
Staphylococcus pyogenes albus.	Diplococcus intra-cellularis meningitidis.
Streptococcus pyogenes.	Typhoid bacillus.
Pneumococcus.	Bacillus coli communis.
Micrococcus tetragenus.	Spirillum of Asiatic cholera.
Bacillus diphtheriæ.	Bacillus pyocyaneus.
Bacillus tuberculosis.	Bacillus of influenza.
Bacillus of anthrax.	Bacillus of glanders.
Bacillus of tetanus.	Bacillus proteus.
Bacillus aërogenes capsulatus.	Bacillus mucosus capsulatus.
	Bacillus of malignant edema.
	Bacillus of bubonic plague.

Special staining methods for cover-glass preparations will be found in the section on Special Bacteriology.

Examination by Cultures.—The demonstration of the presence of bacteria in a tissue or exudate by means of cultures consists in bringing a small amount of the material to be examined in contact with some solid nutrient substance in which the bacteria will thrive. On this the bacteria by multiplication form masses or colonies visible to the naked eye, and present appearances which enable a practised eye in many cases to recognize the species of the bacteria of which they are composed. Of the solid culture-media described in the preceding section, the *coagulated blood-serum* is distinctly the best to use for the demonstration of the presence of bacteria in routine pathological work, because certain of the most important pathogenic bacteria grow better upon it than upon agar-agar or similar media. The other media have important uses in the study of the bacteria after their isolation from the tissues, and in certain instances special media are to be used, as will be pointed out in the following pages. The blood-serum medium here described has been found entirely suitable for the isolation of the bacillus tuberculosis from tubercular lesions, which proves its efficiency as a culture-medium.

Method of Preparing Cultures on Blood-serum.—The preparation of cultures on the coagulated blood-serum consists in distributing over the surface of the medium in a test-tube as much of the tissue or other material as will adhere to the end of a piece of stiff platinum wire hammered flat at the end. The wire is fixed in the end of a glass or metal rod, and should be about 8 cm. long. It should have a rounded spatula-like extremity, and should be thick enough not to bend easily. In making cultures from clinical material, the platinum loop may be used for fluids.

The "platinum wire" or "loop" consists of a piece of platinum wire of about 22 gauge, 2½ to 3 inches long, fixed in the end of a small glass or metal rod 8 or 10 inches long. It is often of great convenience to have two of these instruments, one with the wire curled into a simple loop about 1 to 2 mm. in diameter at the free end, and the other a straight wire with the free extremity hammered flat into a very small spatula. The latter is of great utility in picking up minute portions of bacterial colonies.

Both this instrument and the stiffer wire, above mentioned, should be heated to a red heat in a flame immediately before using, in order to destroy any bacteria that may be upon them.

If the material is on a "swab," the surface of the blood-serum or other media may be conveniently inoculated directly by gently rubbing the swab over it. In this case it is usually best to make a dilution or two by means of the platinum wire, as described below, especially if there be a large amount of material on the swab or if the cover-glass examination has shown that a large number of bacteria are present. In any case it is important that the infected material be spread over all of the surface of the medium, and not in the form of one or two narrow streaks.

It is, of course, essential that the material brought in contact with the culture-medium should be free from bacteria not originally present in it, or that it be not contaminated with bacteria from outside sources. Therefore, in taking material from the interior of organs and tissues the surface is first sterilized by searing it with a hot knife, such as an ordinary case-knife, which has been heated in the Bunsen flame, and then, through a small incision made with another hot knife in this seared or sterilized area, the material from the interior is collected on the end of the platinum wire, which has also been previously heated in the Bunsen flame to sterilize it, and then cooled either by plunging it in the water of condensation of the culture-tube for a few seconds or by moving it about in the interior of the tissue.

In the case of exudations on free surfaces, however, this searing is impossible, and therefore care should be exercised at the autopsy not to contaminate any such exudate by handling before the material for culture has been obtained with the platinum wire. The material thus secured is then transferred by means of the platinum wire to the surface of a blood-serum culture-tube, and the infected wire gently rubbed over *all* of the surface of the culture-medium, avoiding, however, the breaking of the surface. *It is important that the material be well distributed over the nutrient surface.* If the material is suspected of containing a large number of bacteria, as in the case of suppurations or acute inflammatory lesions, a second tube should be inoculated from the first one by touching the platinum wire, previously sterilized and cooled, to the infected surface of the first tube, and then gently rubbing the infected wire over the surface of the second tube. This operation is called "diluting." The object of this is to obtain, after the development of the culture, a sufficiently small number of colonies in the second tube, so that they may be discrete—*i. e.* separated from one another—and thus be enabled to exhibit their characteristic appearances, which are largely lost when the colonies are so numerous as to be confluent.

If thought desirable, a third tube may be similarly inoculated from the second, but this is rarely necessary. In making these "dilutions" it is well to cool the platinum wire in the water of condensation of the sterile tube before touching it to the infected surface of the other tube. As a rule, one tube will be sufficient to obtain discrete colonies from organs or tissues in which no suppurative or exudative condition is present.

Anaërobic cultures are indicated in certain cases. For anaërobic methods, see pp. 118, 119.

After the manner above indicated cultures are to be made at the autopsy as a matter of routine from the *blood of the heart*, from the *liver*, the *spleen*, the *lung*, and the *kidney*. Cultures are also to be made from any acute inflammatory lesion in any situation.

As each culture-tube is infected it is to be labelled with the name of the organ or of the material from which it was infected, and with the date. For this purpose small paper labels coated with mucilage are used.

The culture from the blood of the heart should be made before the removal of that organ from the body, by searing the right ventricle and then puncturing it with a sterilized knife to admit the platinum wire. The amount of blood used for the culture should be as much as will adhere to the platinum wire. Cultures from vegetations in acute endocarditis are not usually of much value unless they are sufficiently large to enable a sterilization of their surface to be effected and material for culture secured from their interior.

Cultures from the Blood during Life.—With a sterilized hypodermic syringe secure a cubic centimeter of blood from one of the large veins at the flexure of the elbow. Mix this immediately with one or two tubes of fluid agar-agar which has been kept at hand at a temperature of about 40° C. and form a "slant," or make a Petri plate of the mixture. (For keeping the agar-agar tubes at the desired temperature an ordinary cup or similar vessel filled with water of the proper temperature will suffice.)

When the "slant" or "plate" has become solid it is to be placed in the incubator for development. Before puncturing the vein the skin over it should be thoroughly cleansed with alcohol and ether, but no antiseptics, such as carbolic acid or corrosive sublimate, should be used. The syringe should be thoroughly sterilized by steam for half an hour, and should be brought to the patient in a sterilized test-tube, from which it should be removed only when it is to be used.

Intraperitoneal inoculation of mice may also be made with ½ to 1 c.c. of the blood. This may give rise to streptococcus or pneumococcus septicemia.

All cultures except gelatin cultures are to be placed, as soon as made, in the incubator or thermostat, where they are conveniently kept in small tin cups.

The Inoculation of Animals.—The inoculation of animals directly with pathological material is often of important diagnostic value. In routine pathological work it is mainly useful in determining the presence of the pneumococcus or of the bacillus tuberculosis when the ordinary methods are considered inadequate.

The methods of inoculating animals are described on pp. 113–118.

III. THE METHODS OF STUDYING BACTERIA IN CULTURES.

THE cultures made as described in the last section, having been in the incubator for eighteen to twenty-four hours, are next to be studied, and the identity or diagnosis of the bacteria whose colonies have grown out upon them is to be established. The identification of the infecting bacteria present in most cases may be made from a consideration of the size, color, and general appearance of the colonies as they appear on the surface of the blood-serum when taken in connection with the morphology of the bacteria composing them. In some cases, however, this may not be sufficient evidence upon which to base the diagnosis, and it may be necessary to obtain further facts in regard to a given organism in order to identify it with a sufficient degree of certainty. Thus it may be necessary to observe the appearances of its growth in pure culture in various media, and to ascertain whether it produces certain chemical changes in the media by its growth. Its ability to grow with or without oxygen, its reaction toward staining agents, whether it has independent motion or not, and its effects upon animals by inoculation, are also points which may have to be determined to enable one to make a positive diagnosis of the species to which the organism belongs. Therefore, in the study of the colonies of the bacteria which have developed in the cultures a familiarity with certain fundamental bacteriological methods is necessary. These will be described in this section.

1. COVER-GLASS PREPARATIONS FROM CULTURES.

A minute proportion of a colony or bacterial growth, the component organisms of which are to be examined, is picked up on the end of the platinum wire, which has been previously heated in the Bunsen flame and cooled, and is thinly distributed on the surface of a cover-glass by gentle movements of the platinum wire. It is very important that the bacteria should be more or less separated from one another in places, so that a good view of the individual organisms may be obtained. This can often best be effected by placing a minute drop of water on the cover-glass first, and then moving the infected end of the platinum wire back and forth through this. The preparation is then dried between the fingers over the Bunsen flame, and next, having been grasped with the Cornet forceps, is to be rapidly passed three times through the Bunsen flame. While still held by the forceps, it is then stained by covering it from a dropping-bottle with the staining solution, and washed in water or submitted to any other manipulation which may be required.

The staining solutions ordinarily employed are carbolic-fuchsin, aniline-gentian-violet, and Löffler's alkaline methylene-blue. If methylene-blue be used, it is best to steam the preparation in staining for about ten seconds, but in the case of the other stains this is not necessary, for they stain deeply almost immediately, so that the staining solution need not remain longer in contact with the bacteria than a few seconds. After staining, the cover-glass is to be washed in water and mounted for examination, as described on page 92 in the case of cover-glass preparations from tissues.

Gram's method of staining may also be used for cover-glass preparations from cultures. As has been pointed out elsewhere (see page 94), this method does not stain all species of bacteria, but some species are stained by it and others are not. This fact is sometimes useful in aiding in the identification of a given organism, and as enabling one to recognize the presence of a few Gram-staining bacteria among a large number of others which are unstained.

In this connection it should be pointed out that bacteria which are stained by this method, when taken from cultures a few days old, may not be stained by it if taken from older cultures. Therefore, Gram's method, if used as a means of differentiation, should be applied only to bacteria in actively growing cultures.

Bacteria stained by this method have a blue-black color.

For a list of bacteria that stain by Gram's method see page 95.

The Staining of Spores.—Spores take up the anilin dyes with difficulty, probably owing to their dense protective envelope. When once stained, however, they do not give up their color easily, and resist decolorizing agents. The cover-glass preparations should be thinly spread.

Abbott's Method.—1. Stain the cover-glass preparation deeply with methylene-blue, heating repeatedly until the staining solution boils, but do not boil continuously, during about one minute.

2. Wash in water.

3. Wash in 95 per cent. alcohol containing 0.2 to 0.3 per cent. hydrochloric acid.

4. Wash in water.

5. Stain for eight to ten seconds in aniline-fuchsin solution.

6. Wash in water and mount.

The spores are stained blue and the bodies of the bacteria red.

Moeller's Method.—1. Wash the cover-glass preparation in chloroform for two minutes.

2. Wash in water.

3. Treat with 5 per cent. solution of chromic acid one-half to two minutes.

4. Wash in water.

5. Stain with carbol-fuchsin, heating slowly until the fluid boils.

6. Decolorize well in a 5 per cent. solution of sulphuric acid.

7. Wash in water.

8. Stain in aqueous solution of methylene-blue (1 gram to 100 c.c.) thirty seconds. The spores will be red, the bodies of the bacteria blue.

The preliminary treatment with chloroform is to cleanse the preparation.

Fiocca suggests the following rapid method: "About 20 c.c. of a 10 per cent. solution of ammonia are poured into a watch-glass, and ten to twenty drops of a saturated aqueous solution of gentian-violet, fuchsin, methylene-blue, or safranin added. The solution is warmed until vapor begins to rise, then is ready for use. A very thinly-spread cover-glass, carefully dried and fixed, is immersed for three to five minutes (sometimes ten to twenty minutes), washed in water, washed momentarily in a 20 per cent. solution of nitric or sulphuric acid, washed again in water, then counterstained with a watery solution of vesuvin, chrysoidin, methylene-blue, malachite-green, or safranin, according to the color of the preceding stain. This whole process is said to take only from eight to ten minutes, and to give remarkably clear and beautiful pictures."

The Staining of Flagella.—All motile bacteria are provided with delicate wavy, hair-like prolongations of their protoplasm, called flagella, which are of comparatively great length. These flagella are the locomotor organs of the organism. The number of them attached to each individual varies to a considerable extent with the species of the bacteria. Thus the individuals of some species have but one flagellum, while the individuals of other species may have few or many springing from all parts of the organism.

The flagella are not rendered visible by the ordinary methods of staining, but special methods are necessary for their demonstration. These methods depend essentially upon the use of a mordant, which causes the flagella to take up the stain.

The cover-glasses must be absolutely free from grease in these methods, so that the watery fluids may be spread evenly over them and not run into patches. The cover-glasses may be prepared by warming them in concentrated sulphuric acid for a time, washing them in water, and keeping them in a mixture of equal parts of alcohol and strong ammonium hydroxid solution.

When used they are to be dried on a cloth which has previously been soaked in ether and allowed to dry, in order that it may contain no trace of fat. Another way to treat the cover-glasses is to take them from alcohol, dry them with a clean cloth, and then heat them by means of the cover-glass forceps in the Bunsen flame to burn off any fat or grease.

The bacteria must be distributed upon the cover-glass well separated from one another in these methods. They should not be subjected to too much manipulation in doing this, for the flagella are readily broken off. A good way is to make a dilute suspension of the bacteria in distilled water, and place one or two loopfuls of this on the cover-glass, not spreading with the loop, but making the suspension flow over the surface by inclining the cover-glass.

Another way is to place two drops of water on a cover-glass—to draw the infected wire once through one of them across the surface, and then once through the other drop, thus making two streaks. This subjects the bacteria to less manipulation and gives a good distribution in places.

The cover-glasses prepared as above indicated are to be allowed to dry in the air, and are then to be heated for a few seconds over a flame while held between the fingers. They are then ready to be stained by any of the methods given below. The cultures used for the preparations should not be older than eighteen to twenty-four hours. Solid culture-media, such as agar-agar, should be employed.

Löffler's Method.—Treat the preparation for about one minute with the freshly filtered mordant solution, which is—

Aqueous solution of tannic acid (20 grams tannic acid to 100 c.c. water),	10 c.c.;
Cold saturated solution of ferrous sulphate,	5 c.c.;
Saturated aqueous or alcoholic solution of gentian-violet or fuchsin,	1 c.c.

The cover-glass is to be covered with this while held with the cover-glass forceps, as in ordinary methods of staining. The mordant, thus placed on the cover-glass, may be gently heated by holding the preparation high over the flame for a period of about one minute, but it must not be boiled. After this the preparation is to be washed in water, and then stained with a freshly prepared and filtered solution of aniline-gentian-violet or aniline-fuchsin, with gentle heating for thirty to sixty seconds. It is then again washed in water, and mounted in water or balsam for examination.

In using this method, as well as others, an important thing to avoid is overheating. The mordant may be freshly mixed every time or kept indefinitely for use.

The ferrous sulphate solution should always be freshly prepared, for it rapidly decomposes. The solution of tannic acid keeps well, however.

The addition of varying quantities of acids or alkalies for different species of bacteria, as recommended by Löffler, is not necessary.

Pitfield's Method as Modified by J. Blackburn Smith.—The mordant is prepared as follows:

A saturated solution of mercuric chlorid, made by boiling, is poured while still hot into a bottle in which crystals of ammonia alum have been placed in quantity more than sufficient to saturate the fluid. The bottle is then well shaken and allowed to cool. To 10 c.c. of this fluid 10 c.c. of a freshly made 10 per cent. solution of tannic acid are added and 5 c.c. of carbol fuchsin solution. After mixing, filter. This mordant will keep.

In staining, the mordant is filtered on to the cover-glass preparation, which is heated until steam is given off during about three minutes. Boiling is to be avoided. The preparation is then washed in distilled water and is stained in a mixture of 1 c.c. of a saturated alcoholic solution of gentian violet and 10 c.c. of a saturated solution of ammonia alum. This mixture is filtered on to the preparation.

Bowhill's Method.—Stain the preparation in the following solution for ten to fifteen minutes, slightly warming:

Saturated alcoholic solution of orcein,	15 c.c.
Aqueous solution of tannin, 20 : 80,	10 c.c.
Distilled water,	30 c.c.

This mixture is to be filtered before using. The saturated alcoholic solution of orcein should be at least ten days old.

Williams' Method.—This is a modification of van Ermengem's method along the lines of the modification of Hinterberger and others. It has been adopted by Dr. Hugh Williams after a large experience with various methods in the Laboratory of the Massachusetts General Hospital.

The method is capable of giving black bacteria and flagella, with little or no precipitate. The method is as follows:

1. Cover the cover-glass with a mordant consisting of

Alumnol,[1] 1 per cent. solution,	1 part;
Osmic acid, 2 per cent. solution,	1 part;
Tannin, 20 per cent. solution,	3 parts.

Shake the mixture, and add three drops of glacial acetic acid, and again shake.

2. Apply the mordant less than one minute without heating. Wash thoroughly in water.

3. Cover the preparation, during about one minute, with a 1 per cent. solution of silver nitrate to which sufficient ammonium hydroxid has been added to keep the silver in solution.

4. Wash in water.

5. Wash with 0.6 per cent. solution of sodium chlorid.

6. Flood the preparation with a 30 per cent. solution of ammonium hydroxid, and immediately wash in water.

7. Apply a few drops of Ortol photographic developer. The directions for making up this developer come with the Ortol.

8. Wash in water.

9. Cover with a 1 per cent. solution of gold chlorid during a few seconds.

10. Wash in water, and apply Ortol developer for a few seconds.

11. Wash in water, and cover with a 1 per cent. solution of mercuric chlorid for a few seconds.

12. Wash in water.

13. Apply Ortol developer for a few seconds.

14. Wash in water, and repeat the application of chlorid of gold, the washing, and the application of the developer two or more times. Between the various applications of the chlorid of gold the preparation should be inspected with a high, dry lens to determine the progress of the staining. This is readily done by placing the cover-glass, charged side upward, on a slide. In this way the process of impregnation with gold may be controlled; for the flagella, if stained, may be easily seen with the high-power dry lens.

The preparation is very conveniently held during the process in cover-glass forceps. The washing is best done in a small stream of water from a faucet. The various solutions are conveniently applied from dropping-bottles, see p. 93.

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[1] Farbwerke vorm. Meister Lucius u. Brüning, Höchst a. M., Germany.

It will be seen that the process consists essentially in the impregnation of the flagella with silver, followed by intensification, in the photographic sense, with mercury and gold. The object of the application of the sodium chlorid and ammonia is to remove the excess of silver compounds which adhere to the surface of the cover-glass in spite of washing. This excess of silver compounds is chiefly responsible for the precipitates which appear on the preparation after the intensification. In spite of the application of the sodium chlorid and ammonia solutions, some precipitate will occur if the intensification is pushed too far. On this account it is advisable to observe the progress of the intensification under the microscope as above indicated.

Although this method may appear complicated, in practice it requires but a few minutes to stain a preparation.

2. METHODS OF OBTAINING PURE CULTURES.

When it is desired to obtain a pure culture of bacteria, a colony or a portion of a colony of the organism is secured on the end of the sterile platinum wire, and transferred by this means to the culture-medium in another test-tube. The bacteria thus sown in the fresh culture-medium multiply there, and produce a growth visible to the naked eye which exhibits appearances more or less characteristic of the species. This growth, if the medium be a solid one, will usually be in the form of confluent colonies; if the medium be a fluid one, the growth may appear as a sediment with or without clouding of the liquid, or it may manifest other peculiarities according to the species to which the organism belongs. If other bacteria are present in the culture from which it is desired to obtain material for a pure culture, it is important that the material should be taken from a colony of the organisms which is well separated from other colonies —*i. e.* that the colony should be a so-called "discrete" one.

In transplanting, the culture-tube containing the colony and the culture-tube that is to be infected from it are held side by side in the left hand in a slanting position in such a way as to give a good view to the operator of the surface of the media in each, while the cotton stoppers are removed and held between the fingers of the same hand (Fig. 17). The object of holding the tubes in a slanting position is to offer less chance of contamination from bacteria gaining entrance to the culture-medium from the air.

The platinum wire, which is manipulated by the right hand, is first sterilized by holding in the Bunsen flame until it glows, and then cooled by contact with the media to be infected, after which its free end is carefully brought in contact with the discrete colony or pure culture-growth, and immediately inserted into the sterile tube to inoculate it. The manner of inoculating the sterile culture-medium in the other tube with the infected platinum wire will vary with the form and character of the culture desired.

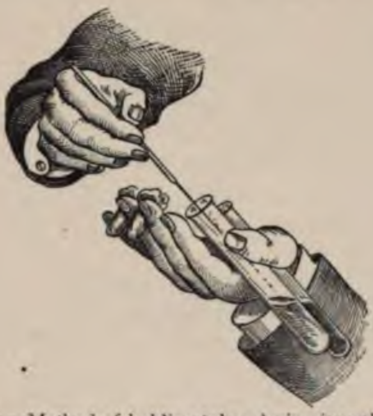

FIG. 17.—Method of holding tubes during inoculation.

If the medium to be inoculated is a fluid one, the wire is simply immersed in it and moved back and forth once or twice. If the medium be a solid one in the form of a slant, the infected end of the wire is drawn over the surface once or twice from the bottom of the slant to its upper end; or if the solid medium in the tube be arranged for a stab culture (see page 77), the infected wire is to be plunged once through the center of the mass to the bottom of the tube. After the tubes have been inoculated as above indicated, the wire is to be immediately withdrawn and the cotton stoppers replaced. They are then to be placed in the incubator for development. Gelatin cultures, however, must not be so treated, but are to be kept at room-temperature, for the heat of the incubator would cause the gelatin to become fluid.

These details as to the manner of manipulating the culture-tubes, cotton stoppers, and platinum wire also apply to the procedure described below.

Method of Isolation of a Bacterium in Pure Culture from a Mixed Growth.—If there is a more or less confluent growth of colonies of various kinds in a culture-tube, and it is desired to isolate a pure culture of one of the species of bacteria present, it is obvious that the first step is to obtain separate or "discrete" colonies of that organism. This is accomplished by securing a minute quantity of the growth on the end of the sterilized platinum wire (preferably from a spot where the organism is prevalent), and distributing this over the surface of a sterile blood-serum tube by gently rubbing the end of the infected wire as thoroughly as possible over it. The wire is then sterilized in the Bunsen flame, cooled in the water of condensation of a second sterile blood-serum tube, next touched to the infected surface of the first tube, and the wire thus infected gently and thoroughly rubbed over the surface of the second. In a similar manner a third tube is then infected from the second, and then all the tubes placed in the incubator for eighteen to twenty-four hours. It is evident that comparatively few bacteria will be sown on the medium of the second tube, and still fewer on that of the third, so that the number of colonies which develop in the

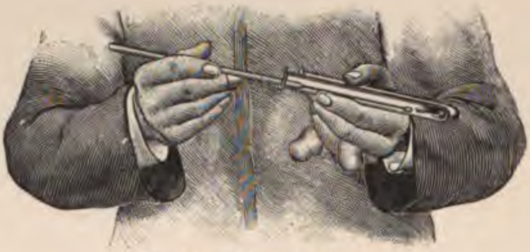

FIG. 18.—Diluting cultures.

second tube will be less numerous than in the first tube, and those in the third tube still smaller in number. Therefore, in either the second or the third tube, or in both, the bacteria sown may be sufficiently few for discrete colonies to develop from them, and among these there may be some composed of the bacterium which it is desired to isolate. From one of such discrete colonies pure cultures may then be prepared as described above. The second and third tubes used in this method are called "dilutions." The details of the manner of manipulating the tubes, etc. in this method may be understood from the description given on page 106 and from Fig. 18.

The Plate Method of Petri.—Another method for obtaining discrete colonies of an organism from a mixed growth of several species is that known as the plate method of Petri. This is a modification of the original complicated method of Koch.

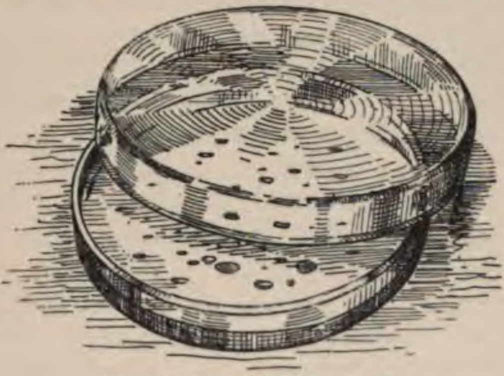

FIG. 19.—Petri dish with colonies.

The method consists in making "dilutions" in melted agar-agar or gelatin tubes, and then pouring the infected medium into shallow glass dishes (Fig. 19) previously sterilized, in which it is allowed to solidify. A few bacteria are thus distributed throughout a thin layer of culture-medium in the "dilutions," and the colonies which develop from them are then more or less separated from one another, so that pure cultures may be obtained from them. In carrying out this method the procedure is as follows:

Three sterile gelatin or agar-agar tubes are melted by heat and placed in a water-bath warmed to between 40° and 42° C. for several minutes, to bring the culture-medium to this temperature. This temperature is important especially in the case of agar-agar, for it is just above the solidifying point of that medium (38° C.) and yet not injurious to the vitality of the bacteria. The tubes are then infected successively from the bacterial growth or from the pathological material from which it is desired to obtain discrete colonies, in the same manner as described for the method with blood-serum tubes —viz. one tube being inoculated from the growth or tissue, a second tube or dilution from the first tube, and a third tube or dilution from the second tube, the platinum wire being sterilized after each inoculation. For making the "dilutions" a platinum wire bent into the form of a small loop (see page 96) is to be used, and as much of the culture-fluid as will adhere to it used for inoculating. The wire should be moved back and forth several times in the medium of each tube when inoculating it, in order to ensure a good distribution of the bacteria throughout the fluid. The contents of each tube thus inoculated are then poured into sterilized Petri dishes, in which the culture-medium solidifies in a thin layer.

The Petri dishes (Fig. 19) are of clear glass, circular in form, 10 cm. in diameter and about 1 cm. deep. Each is provided with a loosely fitting flat cover of glass. These dishes with their covers are to be sterilized before using by placing them in the steam sterilizer for half an hour or by heating them to 150° C. in the hot-air sterilizer. When cool they are ready to receive the contents of the inoculated test-tubes. In pouring, the cover of the dish is not to be removed any more than is necessary, and it is to be immediately replaced, so that contamination from the air may be better avoided. It is very desirable that there be no dust about the place where the dishes are "poured," and no currents of air.

If agar-agar is used, the dishes thus prepared are to be put in the incubator for eighteen to twenty-four hours as soon as the medium is solid, which it becomes in a few minutes; but if gelatin be used, the dishes are to be set aside in a cool place, free from dust, to solidify, and are then to be kept at room-temperature for several days. Colonies first begin to appear in the gelatin usually after forty-eight hours.

The method of Petri is of great utility in the study of bacteria from the botanical standpoint, for it is especially adapted for the study of the appearances of colonies under the low power of the microscope. It is, however, inferior to the method with blood-serum tubes for routine pathological work, for the following reasons: First: Certain pathogenic bacteria grow only feebly on the culture-media which it is necessary to employ in this method, while they grow comparatively vigorously on blood-serum. Second: The method is complicated and much more troublesome and time-consuming than the simple method described above.

Esmarch's Method of Roll-cultures.—A third method for obtaining discrete colonies is that of Esmarch as modified by Sternberg. It consists in melting and inoculating three gelatin or agar-agar tubes as described for the plate method of Petri, and then distributing and solidifying the infected culture-medium over the inner surfaces of the tubes in the form of a thin layer by rolling the tubes on a block of ice while the medium in them is still fluid (Fig. 20). As in the plate method of Petri, the end in view is the distribution of a few bacteria throughout a thin layer, and the consequent development from them of discrete colonies.

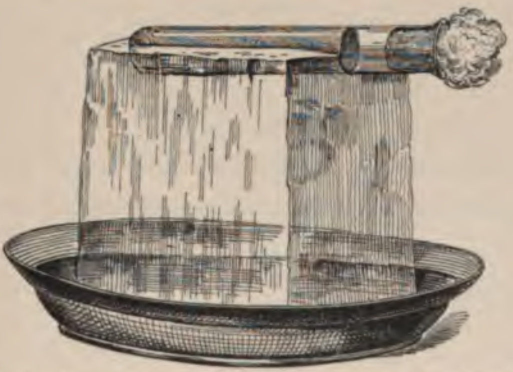

FIG. 20.—Esmarch tube on block of ice (redrawn after Abbott).

The tubes used for this purpose should contain sufficient culture-medium to fill them to a depth of about 2 cm., but not much more than this, for with a greater quantity the preparation of a thin layer is difficult.

In carrying out this method it is best to first make a depression in a block of ice with a test-tube filled with hot water. In this depression the culture-tube containing the fluid is placed in a nearly horizontal position, and rotated rapidly with the fingers until the medium is properly distributed and solid, care being exercised not to permit the medium to come in contact with the cotton stopper. The even distribution of the medium over the inner surface of the tube is best obtained by manipulating the tube while the medium is still fluid, in such a way as to moisten its inner surface up to about 1 cm. of the cotton stopper before proceeding to rotate it on the ice.

Gelatin is the best culture-medium to use in this method. Agar-agar can be used, but has the great disadvantage of readily slipping down toward the bottom of the tube if the tube is placed upright. It is necessary, therefore, to keep the tubes on their sides. The method has little or no advantage over the method of Petri.

The Determination of the Motility of Bacteria.—This is done by observing the individual organisms, unstained, in a drop of bouillon or similar fluid under the oil-immersion lens. For this purpose a so-called "*hanging drop*" is prepared, for which a special form of slide known as a "*hollow slide*" is necessary. The hollow slide is a slide having a shallow circular concavity, about 1 cm. in diameter, ground out in its center (Fig. 21).

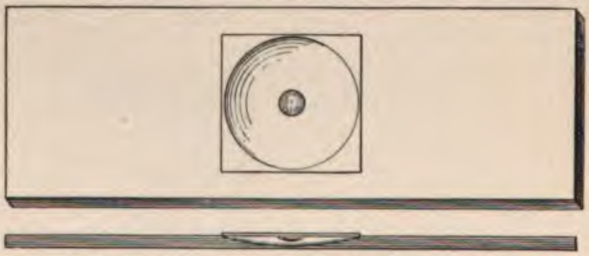

FIG. 21.—The "hanging drop" seen from above and in profile.

In **preparing a hanging drop** the procedure is as follows: A small drop of a bouillon culture or of the water of condensation of a blood-serum or agar-agar slant is placed in the center of a cover-glass by means of the platinum wire.

The cover-glass is then placed, drop downward, over the circular depression in the hollow slide. To hold the cover-glass in its place and to prevent evaporation of the fluid in which the organisms are suspended, a little vaselin is painted around the margin of the depression before placing the cover-glass in position. The hanging drop thus prepared is then examined by focusing upon it with the oil-immersion lens, a small aperture of the iris diaphragm of the condenser being used to render the bacteria visible by refraction. To facilitate focusing, the edge of the drop should be brought into the center of the field of the low-power objective, and then the oil-immersion put in place and focused upon it, the edge of the drop being more readily seen as a sharp line, owing to refraction, than the organisms. Great care is necessary to avoid breaking the cover-glass in the effort to bring the bacteria into view. Hanging drops may also be prepared from suspensions of bacteria grown on solid media, by mixing a portion of the growth with a small quantity of bouillon.

In the study of spore-formation the hanging drop is of great utility. Here the slide and cover-glass must be carefully sterilized before using, the cavity between the cover-glass and the slide well sealed with vaselin, and other precautions taken to prevent contamination of the drop with other bacteria. The preparations may be placed in the incubator or on a "warm stage" and the process of spore-formation followed.

3. THE INOCULATION OF ANIMALS.

The animals ordinarily used in the laboratory are guinea-pigs, rabbits, and mice. The instruments, etc. used in the inoculation of animals should be sterilized beforehand, but strict surgical asepsis is not necessary as a rule.

Guinea-pigs are in most instances inoculated either subcutaneously or into the peritoneum.

Subcutaneous inoculation is effected either by injection with a hypodermic syringe or by the introduction of the material to be inoculated through a small incision in the skin. The best point for subcutaneous inoculation is the tissue of the anterior abdominal wall.

In inoculating, the animal is to be held abdomen uppermost by an assistant, who grasps the neck and fore quarters with one hand and the hind quarters with the other. If the skin is to be incised, the hair about the point of inoculation is to be cut short with a pair of scissors and the skin cleansed with soap and water. An incision is then to be made about 8 or 10 mm. long through the skin, including the subcutaneous tissue, and the superficial tissues separated from the muscle for a distance of 10 or 15 mm. toward one side of the wound by inserting the points of scissors or other instrument, so as to form a "pocket" beneath the skin. In this "pocket" the material for inoculation is introduced, either on the platinum wire (see page 96) or by means of small forceps.

If pieces of tissue are used, it may be well in some cases to close the wound by one or two sutures in order to prevent the extrusion of the material after the release of the animal.

Intraperitoneal inoculation may be performed essentially as above indicated. If the inoculation be by incision, the opening into the peritoneal cavity should be as small as possible, and the wound should be firmly closed with silk sutures in order to prevent extrusion of the intestines.

In inoculating with the hypodermic syringe the needle should not be pushed in too far or the intestines may be wounded. The needle is best introduced a little to one side of, or slightly below, the umbilicus.

Rabbits.—These animals may be inoculated both subcutaneously and intraperitoneally, essentially as described for guinea-pigs.

In lifting or in carrying rabbits from one place to another the animals are to be grasped by the ears. During the operation of inoculating, the assistant grasps the ears with one hand and the hind legs with the other, while the body of the animal rests upon the table, abdomen uppermost. Rabbits held for a few seconds in this position usually become perfectly quiet, and often do not show any evidence of pain during the operation.

Intravenous inoculation is usually done on rabbits, because of the ease with which the needle of a hypodermic syringe may be introduced into the long and prominent marginal vein of the ear. In inoculating in this manner the tip of the ear is held by the thumb and fingers of the left hand, while the right manipulates the syringe, the needle of which is pushed through the skin of the external surface of the ear into the vein which runs along the outer margin of the ear (Fig. 22).

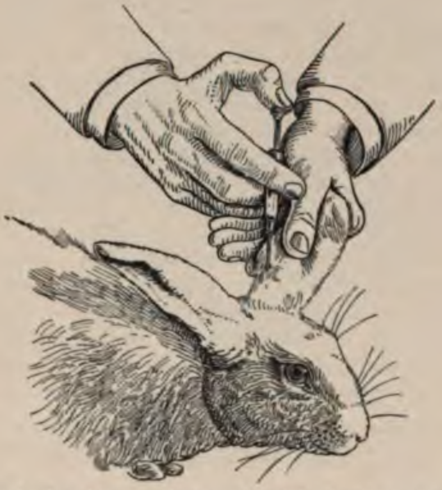

FIG. 22.—Method of making an intravenous injection into a rabbit. Observe that the needle enters the posterior vein from the hairy surface (McFarland).

By the exercise of care and gentleness the animal may be thus inoculated without being held by an assistant, especially if the fur between the ears be stroked for a short time just before the introduction of the needle. In some cases it may be necessary to anesthetize the animal on account of violent struggling. (See below.)

Injection of bacteria into the mesenteric veins by means of the hypodermic syringe, after laparotomy, may be performed both on rabbits and on guinea-pigs. This is to be done under anesthesia. Ether is very satisfactory for this purpose. Guinea-pigs bear it well, but it is to be used with caution on rabbits. With the latter animals death is liable to occur if the ether is "pushed" after complete anesthesia is established. Rabbits once thoroughly anesthetized seem to remain so for a considerable time without additional ether being necessary. The incision for this form of inoculation should be in the lower half of the abdominal wall in the median line, for in this region the coils of the small intestine are most numerous. The length of the incision should be about 2 cm. Several loops of intestine are brought out through the wound, and a mesenteric vein, of the proper size to admit the needle of a hypodermic syringe, is sought for. When found the needle is to be introduced and held firmly in position while an assistant carefully presses inward the piston of the syringe. After the injection of the material the needle is withdrawn, the punctured vein picked up with the artery-forceps, and the vessel tied on both sides of the puncture with silk thread. The loops of the intestine are then replaced and the wound closed in two layers, one consisting of the muscles and peritoneum, the other of the skin. The so-called "button-hole stitch" with silk thread is very well fitted for the closing of the wound.

Little or no aseptic precautions are necessary to obtain primary union in the wound. Before the operation, however, the hair of the region should be cut off close and the skin cleansed with soap and water.

This form of inoculation may be useful in studying the local effects of bacteria upon liver-tissue, for large numbers of them will be lodged in the capillaries of the liver, and microscopical sections of any part of the organ will contain them, so that any local lesion produced by them may be subjected to observation after variable intervals of time.

Mice are usually inoculated subcutaneously at the root of the tail. The animal, manipulated by means of chemists' crucible tongs or a similar instrument grasping his tail, is to be persuaded to crawl into a cylinder of wire gauze, about 8 to 10 cm. long and about 3 cm. in diameter, which is fixed on a small board. The cylinder is open at both ends, and when the mouse has crawled into it—a thing which he will readily do—the end near his tail is bent inward so as to prevent him from backing out of it, while an ordinary small screw-clamp is adjusted firmly to his tail to prevent his escaping through the other end. The animal is thus secured and ready for the operation of inoculation. A more complete form of this apparatus, with a fixed clamp for the animal's tail, is shown in Fig. 23.

In making the inoculation the mouse is pulled backward by the tail until his rump is exposed in the end of the cylinder, and then with small scissors the hair is cut away over a space, approximately 1 cm. square, about the root of the tail. In the center of this a small opening is made through the skin 3 or 4 mm. long with small scissors, and through the opening the points of the scissors are passed anteriorly beneath the skin for a distance of about 1 cm., so as to make a "pocket" or cavity by separating the skin from the muscles. Into the cavity thus formed the material for inoculation is then to be introduced by means of the platinum wire. As a rule, white mice are to be preferred to the wild brown variety, on account of the greater ease with which they may be handled.

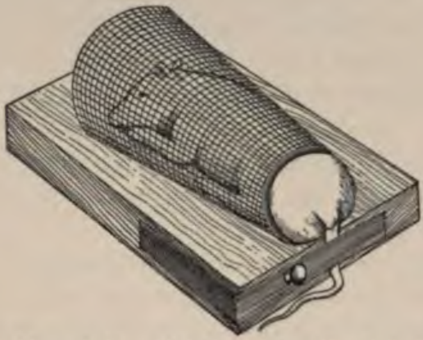

FIG. 23.—Mouse-holder, with mouse in position for inoculation.

Mice may also be inoculated in the peritoneal cavity by introducing a very few drops of a suspension or a bouillon culture of an organism with a hypodermic syringe.

The quantity of bacteria used for purposes of inoculation varies with the organism and with the end in view. In general, it may be said that in inoculating with the growth from a solid medium with the platinum wire one or two loopsful are used. If bouillon cultures are employed, the quantity injected varies from $\frac{1}{10}$ c.c. to 1 c.c. in most cases.

In cases where a "suspension" of the growth on a solid medium is injected the same quantities are used as in the case of bouillon cultures, the density of the suspension depending upon the operator. A "suspension" may be conveniently prepared by pouring 5 or 8 c.c. of sterile bouillon, sterilized water, or 0.6 per cent. sodium chlorid solution (sterilized) into the tube containing the growth upon solid medium, then breaking up the colonies of the growth with the platinum wire, and shaking the tube.

The Care of Animals.—Inoculated guinea-pigs should be kept in boxes or cages so arranged as to permit of cleaning and disinfection. Cages made of a combination of galvanized-iron wire netting and galvanized sheet iron are to be preferred. The bottom of the cage should contain sawdust, and the top may be made to open on hinges. Good dimensions for such cages are 16 inches long, 10 inches wide, and 10 inches high. They may be satisfactorily disinfected, in most instances at least, by washing with boiling water.

Inoculated mice are well kept in large glass jars with perforated covers. A small amount of raw cotton should be provided for bedding.

The "stock" guinea-pigs and rabbits may be kept together in a pen which should have light and ventilation. Guinea-pigs breed readily and their young thrive, but this is not usually the case with rabbits. Mice may be kept for use in a woven-wire cage set in a sheet-iron pan, which will permit of the easy removal of excreta. Some raw cotton should be furnished for bedding. The young of white mice are difficult to raise to maturity.

Food.—Rabbits and guinea-pigs eat the same things. In summer-time, grass, green corn-husks, and green vegetables generally are good food for them. In winter, carrots and oats form a satisfactory diet. Fresh water should also be supplied.

Mice may be fed on stale bread soaked in water, oats, birdseed, and occasionally some cheese. Fresh water should be furnished, and, if possible, a little milk sometimes.

4. CULTIVATION WITHOUT OXYGEN (ANAËROBIC CULTURES).

Of the numerous methods and modifications of methods that have been proposed for the cultivation of anaërobic bacteria, only those are given here which have worked successfully in our hands, or are regarded as the simplest and most practical.

Culture-media for Anaërobic Bacteria.—The agar-agar, gelatin, or bouillon used for the cultivation of anaërobic bacteria should contain 1 per cent. glucose. These media should not be more than two weeks old for the best results.

Their reaction is of the greatest importance, and should be adjusted by titration (see page 83). In the case of gelatin and of agar-agar the reaction should be 1 per cent. or 1.5 per cent. of normal acidity to phenolphthalein. In the case of glucose bouillon, however, a more rapid growth is obtained with a reaction of less than 1 per cent. normal acidity to phenolphthalein. Moreover, glucose bouillon undergoes a spontaneous increase in acidity while kept in stock, and in the course of a few days may become incapable of supporting the growth of an obligate anaërobe unless its reaction be readjusted. This is probably a frequent cause of failure to obtain cultures of obligate anaërobes in bouillon. Therefore, it is of the utmost importance, in working with glucose bouillon, to be sure that it has the proper reaction at the time of its use. In general it may be said that a bouillon adjusted to a reaction of 0.5 per cent. of normal acidity to phenolphthalein will be available for the cultivation of obligate anaërobic bacteria during a week without readjustment of its reaction.

The culture-medium must be thoroughly boiled immediately before inoculation in order to expel absorbed oxygen. It is then to be cooled rapidly by immersing the tube in cold water, and is to be inoculated within a few minutes afterward.

Method of Liborius (Fig. 24).—This consists in cultivating the bacteria in the depths of solid media in test-tubes filled to a considerable height, so that oxygen cannot penetrate to them through the thick layer of medium.

A test-tube is filled about three-quarters full[1] of sterile glucose gelatin or glucose agar-agar, and its contents boiled for a few minutes to expel the excess of oxygen from the medium. The tube is then immersed in cold water to cool its contents rapidly, and then, before the medium becomes solid, the tube is placed in a water-bath at 38° to 40° C. for a few minutes. When the medium may be assumed to have reached this temperature, it is inoculated with the material from which a growth is sought to be obtained, and then rapidly solidified in cold water. The colonies of anaërobic bacteria develop only in the deeper layers of the culture-medium. These colonies may be made accessible for sub-cultures either by breaking the tube or by removing the overlying portions of the culture-media by means of a stout platinum wire, previously sterilized in a flame. For taking out colonies for transplantation, a capillary glass tube, sterilized in a flame, may be found useful in place of the platinum wire. In inoculating the tube, care should be taken to secure a good distribution of the bacteria through the medium by manipulating the platinum wire.

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[1] The tube need not be filled more than half its length.

FIG. 24.—Liborius's method of making anaërobic cultures.

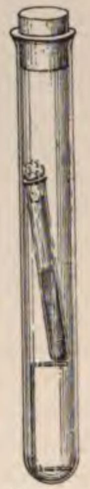

FIG. 25.—Buchner's method of making anaërobic cultures.

This method will be found very practical for obtaining pure cultures from mixed growths if dilutions (see page 110) be made. In making dilutions it is well to use a tube of bouillon or sterilized water for the first tube, thus economizing medium, for the first tube will usually have so many colonies that no colonies suitable for sub-cultures will be available.

The microscopical appearances of the colonies may be studied by placing thin slices of the medium, containing the colonies, on a slide. These slices may be easily obtained with the aid of a stout platinum wire with a flattened end, more or less bent.

Anaërobic bacteria grow readily in "*deep stab*" *cultures.* In these cultures the medium should fill the tube to almost half its height at least. After inoculation some melted medium may be poured in so as to fill the tube to an additional height of some centimeters, but this is not necessary.

Esmarch's Method.—This consists in preparing an Esmarch roll-culture (see page 111), of the organism, and, while the layer of glucose gelatin is still cold from the ice, filling the tube with liquefied gelatin and solidifying this rapidly in cold water. The end of the tube is then to be sealed airtight. The colonies develop in the layer of gelatin close to the side of the tube, and thus can be readily studied with low magnifying power.

Simple Anaërobic Plate-cultures.—These are prepared like the ordinary Petri plate-cultures (see page 109) except that the melted culture-medium is poured into the upturned larger dish, or cover, of the pair, while the smaller dish is then placed, bottom surface downward, in the melted culture-medium, and allowed to settle by its own weight into the fluid medium. The dishes are not disturbed until the medium has hardened. Sufficient medium should be used to fill the space between the sides of the dishes. This quantity will be about 10 c.c. By slightly inclining the smaller dish in placing it in the melted medium, air-spaces can be easily avoided.

By this method the colonies develop in a thin layer of culture-medium enclosed between glass surfaces. The method gives a good chance to study the microscopical characters of the colonies. Surface colonies are, of course, not obtained by this method. The colonies are easily made accessible for transplantation by separating the dishes from one another. The layer of culture-medium will adhere to one dish or the other.

In order to avoid contamination, the dishes should be arranged in the manner above described during their sterilization previous to using.

Buchner's Method.—This method consists in cultivating bacteria in an atmosphere from which the oxygen has been absorbed by a mixture of alkali and pyrogallic acid. Tube-cultures, or cultures in Petri dishes, may be used. They should be placed in some form of a glass chamber, which is closed air-tight, along with the necessary quantity of alkali and pyrogallic acid mixture. In preparing the apparatus, the pyrogallic acid (in powder) is placed first in the chamber along with the culture tubes or plates, then the necessary quantity of a solution of potassium hydroxid (1 : 10) is run in, and the chamber quickly closed. For single tube-cultures a large test-tube provided with a tightly-fitting rubber stopper, which is sealed in position with wax, may be used for the air-tight chamber (see Fig. 25.) The culture-tube is to be elevated above the surface of the reducing mixture by means of a bent wire.

If a number of tube-cultures or Petri plate-cultures are desired, the glass chambers known as Novy's jars are very satisfactory to use. The joints of this apparatus should be well smeared with vaselin. To avoid breakage the test-tube containing the inoculated culture-medium may be held in a beaker, with some cotton at the bottom, while in the apparatus. Petri plate-cultures may be placed one above another in the jar, the bottom plate being supported above the level of the reducing fluid by some sort of wire frame.

It is necessary to seal up the apparatus quickly in order to obtain the full benefit of the oxygen-absorbing power of the pyrogallic acid. The quantity of pyrogallic acid employed should be about 1 gram for each 100 c.c. of air-space to be exhausted of oxygen, and for every gram of pyrogallic acid 10 c.c. of the solution of potassium hydroxid should be used.

Wright's Method.[1]—The method depends upon the absorption of oxygen by an alkaline solution of pyrogallic acid as in the well-known method of Buchner. It is applicable to cultures in test-tubes, and probably also to cultures in flasks. The details of the method are as follows:

After the culture-medium in the test-tube has been inoculated, the cotton stopper is thrust sufficiently far down into the test-tube so that the upper end of the cotton stopper lies about 1 cm. below the mouth of the test-tube. It is usually desirable to cut off a part of the protruding portion of the cotton before doing this. Next there is run into the cotton of the stopper from a pipette a small quantity of a watery solution of pyrogallic acid and of sodium hydrate solution. Then the tube is immediately closed air-tight by firmly inserting a rubber stopper in its mouth. The culture is then all ready to be set aside for development.

The watery solution of pyrogallic acid is made by dissolving in a convenient quantity of water an approximately equivalent quantity or bulk of pyrogallic acid.

The solution of sodium hydrate consists of one part of sodium hydrate in sticks and two parts of water.

For cultures in test-tubes, of a size 6 by ¾ inches, ½ of a cubic centimeter of the aqueous solution of pyrogallic acid and 1 cubic centimeter of the sodium hydrate solution, both measured roughly, have been found sufficient quantities of these solutions to be placed in the cotton stopper, as above described. The stopper must be of absorbent cotton.

The solution of pyrogallic acid should be freshly prepared, and should be run into the cotton stopper before the solution of alkali. The pipette should be rinsed with water before using it to run in the solution of alkali. The rubber stopper is to be inserted as soon as possible after the solution of alkali has been run in. For this reason it should be kept at hand during the manipulations above described.

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[1] *Journal of the Boston Society of Medical Sciences*, Dec. 4, 1900.

It may be thought that there is danger of contaminating the culture-medium from the alkaline pyrogallic acid mixture running down the sides of the tube. This does not occur, because the mass of the cotton stopper is sufficiently large to absorb completely the quantity of fluid in it, with a good margin to spare.

This simple method has given satisfactory cultures with three examples of the tetanus bacillus obtained from cases of tetanus in the Massachusetts General Hospital and with three other obligate anaërobic bacteria. It can be applied to all forms of test-tube cultures, both in solid and fluid media, including Esmarch roll-cultures. Although it has not been tested on cultures in flasks, there seems to be no reason why it should not be used for this purpose. In applying the method to Esmarch roll-cultures the mixture of pyrogallic acid and alkali should be placed in the cotton, and the rubber stopper inserted before the tube is rolled on the ice. Glucose agar readily lends itself to Esmarch roll-cultures if the tubes are kept in a slanting position during growth.

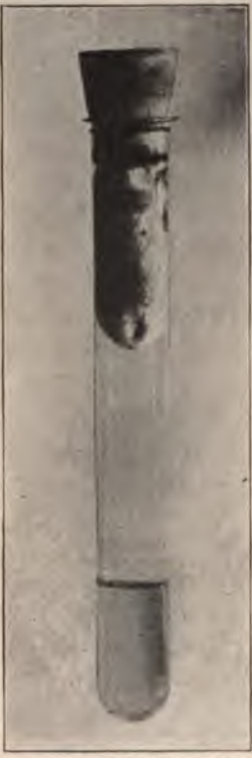
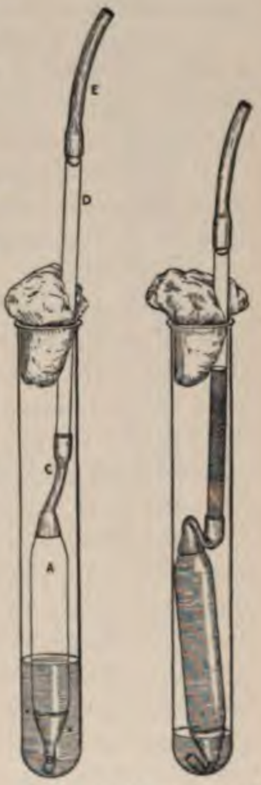


FIG. 26.—Wright's method for the cultivation of anaërobes.

The accompanying photograph shows the appearance of a bouillon tube prepared according to this method (Fig. 26).

A Simple Method for Anaërobic Cultivation in Fluid Media (J. H. Wright).—The method here described has been successfully used for the cultivation of the tetanus bacillus.

The apparatus consists of a simple arrangement of glass and rubber tubes enclosed in an ordinary test-tube with a plug of cotton inserted in its mouth as in an ordinary culture-tube. The construction of the apparatus will be plain from a consideration of Fig. 27.



FIGS. 27, 28. — Wright's method of making anaërobic cultures in fluid media.

A is a glass tube somewhat constricted at each extremity; B and C are short pieces of small rubber tubing; D is a glass tube in the upper extremity of which a small plug of cotton is inserted; E is a piece of rubber tubing. The test-tube contains a quantity of the culture-fluid as indicated by the figure.

When it is desired to make an anaërobic culture, the fluid in the test-tube is inoculated in the usual way. The fluid is then sucked up into the system of glass and rubber tubes to a level above the rubber tube C. When it has reached this level, the rubber tube E is compressed between the fingers to prevent the down-flow of the fluid, and the system of tubes is then pushed downward in such a way as to bend the rubber tubes B and C in the manner shown in Fig. 28. If the test-tube and the inner tube system are of suitable size, the rubber tubes mentioned will remain in this bent position. The fluid in the tube A is thus contained in a water-tight space, because the rubber tubes B and C, when bent to the angle shown in Fig. 28, are thereby closed water-tight. That a flexible rubber tube does so close itself when bent to a certain angle is a matter of common observation.

Cover-glass preparations may be made from the culture-fluid by straightening out the system of tubes and allowing the fluid in them to flow out into the test-tube, where it is accessible to the platinum loop in the usual way.

The advantages of this method are:

1. Its simplicity and the easy construction of the apparatus.

2. The possibility of keeping any number of pieces of the apparatus on hand sterilized ready for use as ordinary bouillon tubes are kept on hand.

3. It offers a means of determining whether a given bacterium grows better under aërobic or anaërobic conditions, by comparing the growth in the fluid outside in the test-tube with the growth of the fluid in the inner tube A.

4. It makes it possible for the user to form some idea of the purity or impurity of the culture with the naked eye, for if one is working with strict anaërobes the lack of bacterial growth in the fluid outside in the test-tube is a good sign of the absence of contamination with aërobic bacteria.

In using this method it is, of course, necessary that most of the air in the culture-fluid be expelled before it is inoculated. This is easily done by boiling the culture-fluid over the flame of a Bunsen burner without removing the inner system of tubes, and then cooling the apparatus by placing it in cold water.

The sterilization of the apparatus is effected by steam, care being taken that the rubber tubes be not bent in the position shown in Fig. 28 during the sterilization, for the heat may set them permanently in this position.

Bouillon Cultures under Hydrogen.—These are prepared by displacing the air in an ordinary culture-tube or other vessel containing glucose bouillon by hydrogen, and hermetically sealing the tube. The bouillon-tube is closed by a rubber stopper through which two small glass tubes pass, one running to the bottom of the tube, the other extending just inside the rubber stopper.

The tubes outside the stopper are bent at right angles, and then, beyond the bends, are constricted by drawing out while hot, so as to be readily sealed with the Bunsen flame. In the outer ends of the tubes loose plugs of cotton are placed, and the apparatus thus prepared is sterilized by steam for half an hour. After sterilization and cooling the bouillon is infected with the organism, the stopper and its glass tubes being removed and replaced with care to avoid contamination. The stopper is then sealed in position with paraffin or sealing-wax, after which hydrogen from a hydrogen generator is passed through the longer tube leading into the bouillon, and thus displaces the air through the shorter tube. After the hydrogen has flowed for five minutes the glass tubes are quickly sealed with the Bunsen flame at the constrictions, and the culture thus prepared placed in the incubator. In passing the hydrogen through the apparatus the cotton plugs must of course be kept in position.

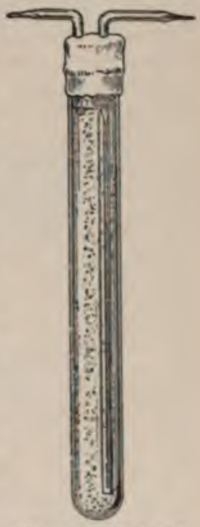


FIG. 29.—Fränkel's method of making anaërobic cultures.

The generation of hydrogen is best effected by means of the well-known apparatus of Kipp. It is important that no oxygen be present in the gas, or an explosion may occur during the sealing of the tubes in the Bunsen flame.

Pure granulated zinc and pure sulphuric acid of 25 to 30 per cent. strength should be used. Before running the gas into the test-tube or other apparatus the reservoir of the generator should be allowed to fill with gas, and this then allowed to escape into the air by opening the outlet, care being taken that no flame be near by. This should be repeated, and then a sample of the hydrogen collected in an inverted test-tube by displacement of water in the well-known way. The same should then be ignited, and if no explosion occurs the hydrogen is fit to use.

The hydrogen should be washed to insure the removal of impurities from it by passing it through two washing-bottles before it is passed into the culture apparatus. One of these washing-bottles should contain an alkaline solution of pyrogallic acid, the other a 10 per cent. solution of acetate of lead. These may be easily constructed out of two large test-tubes provided with rubber corks through which the necessary glass tubes pass.

Cultures under hydrogen on solid media may also be made in a manner similar to that described for bouillon cultures.

An ingenious application of the method above described to

Esmarch roll-cultures under hydrogen is the so-called **Fränkel's method** (Fig. 29). This consists in preparing dilutions in melted gelatin tubes, passing hydrogen through the melted gelatin in the tubes as in the case of bouillon, so as to displace all of the air, sealing the tubes, and then making Esmarch rolls on ice (see page 111). While filling with the hydrogen the tubes are to be kept in a water-bath at about 35° C., in order that the gelatin may remain fluid. All of the precautions mentioned in the case of bouillon are to be observed in order to prevent contamination.

Plate-cultures by Petri's method, or ordinary tube-cultures, may also be grown by placing the plates or tubes in a vessel so constructed that hydrogen may be passed into it until all the air is displaced, and the vessel is then hermetically closed. The apparatus of Novy, now on the market, is well adapted for this purpose.

Before passing in the hydrogen a plug of cotton should be placed in each of the tubes of the apparatus, to prevent the access of bacteria to the interior. The entire apparatus should also be sterilized by steam before the plates are placed in position and before the hydrogen is run in.

The hydrogen should be passed through the Novy jar until a sample of the gas that issues from it is found to be free from oxygen by the test just described. Then the valve of the jar is to be closed for five or ten minutes, after which time more hydrogen is to be passed through the apparatus. When the outcoming gas has been proved to be free from oxygen, the valve is to be permanently closed and the apparatus set aside for development of the cultures.

Cultures in Vacuo.—These are very satisfactorily made by placing the culture-tubes or culture-plates in the Novy jars together with an alkaline solution of pyrogallic acid, as described under Buchner's method, and then exhausting the air contained on the tubes by means of a water-vacuum pump such as chemists use. The pyrogallic acid will thus absorb the oxygen which cannot be removed by the pump. The Novy jars are very good to use for this purpose, because they are provided with suitable air-tight valves.

IV. SPECIAL BACTERIOLOGY.

The number of species of bacteria of pathogenic significance which are *commonly* encountered in pathological processes in man is a small one. These comprise the staphylococcus pyogenes aureus, the streptococcus pyogenes, the pneumococcus, the bacillus coli communis, the typhoid bacillus, the bacillus diphtheriæ, and the bacillus tuberculosis.

It is with infections with these few species that the pathologist is most frequently concerned, and the determination of the presence of these alone comprises by far the greater part of the bacteriological work which he is called upon to do.

In the following descriptions of the important pathogenic bacteria which are concerned in human pathology the main object will be to give those characteristics which will serve for their identification, rather than an exhaustive consideration of their various properties and modes of growth.

Staphylococcus Pyogenes Aureus.[1]—The colonies on blood-serum are golden yellow in color. They are rounded, shining, slightly elevated, and may attain a diameter of 2 mm. or more after remaining for thirty-six hours in the incubator. The color of the colonies varies from a pale yellow to a deep orange. Young colonies may be creamy white, becoming yellow later.

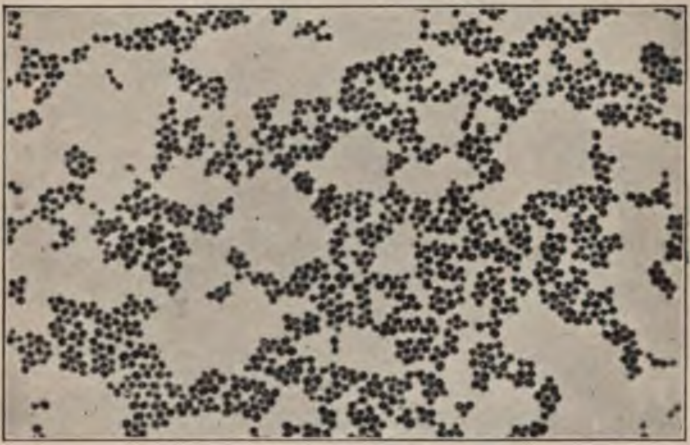


FIG. 30.—Staphylococcus pyogenes aureus from a culture; × 2000 (Wright and Brown).

Morphology.—Rather small cocci, frequently arranged in masses or clumps.

Stained by Gram's method.

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[1] J. Rosenbach: *Mikroörganismen bei den Wundinfectionskrankheiten des Menschen*, Wiesbaden, 1884.

Gelatin Stab-culture.—Growth along the line of stab, followed by liquefaction in funnel form, with yellow sediment and clouding of the liquefied medium (Fig. 31).

Potato.—Yellow confluent colonies.

Agar-agar Slant.—Rather broad shining streak with sharply defined margins, at first white in color, but later becoming yellow.

Bouillon.—Densely clouded. A yellowish sediment is formed, and sometimes a thin pellicle is seen on the surface.

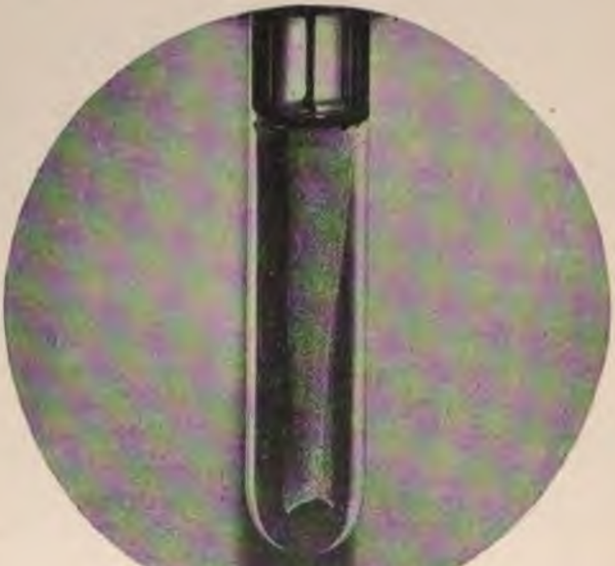

FIG. 31.—Staphylococcus pyogenes aureus: stab-culture three days old in gelatin (Fränkel and Pfeiffer).

Litmus-milk.—Turned pink and coagulated.

Pathogenesis.—When inoculated into the circulation of a rabbit death follows in from eighteen hours to three days in the case of virulent cultures. Not all specimens of this organism are virulent. The lesions produced in the rabbit by inoculation in the ear-vein in typical cases are abscesses with infarctions in the kidneys, and miliary abscesses in the myocardium, diaphragm, and voluntary muscles. In the kidneys lines of necrosis with purulent infiltration, mainly in the pyramids, are frequently observed. This organ is the one most constantly affected. The number and extent of the lesions vary in different animals and with different cultures. They are best developed in animals which survive about three days. In animals which succumb after eighteen hours no macroscopic change may be apparent. On microscopical examination of the kidneys, however, small areas of necrosis will usually be found, mainly in the pyramids, surrounding masses of cocci. In the kidneys of animals which survive longer all the grades of invasion of these necrotic areas by leucocytes, up to regular abscess-formation, may be traced. By cultures the organism will be found in large numbers in the kidneys and urine of the rapidly fatal cases, and in smaller numbers in the other organs and blood of the heart.

Occurrence.—The staphylococcus pyogenes aureus is found most commonly in pus-formations of a circumscribed character and also in a large number of pathological conditions, of which only the more important will be mentioned here.

These are as follows: Osteomyelitis, peritonitis, pleuritis, endocarditis, meningitis, broncho-pneumonia, and puerperal septicemia. It may also be found in the blood of the various internal organs at autopsies in cases in which a suppurative or other acute inflammatory process is present anywhere, with or without metastatic abscess-formation. The organism also occurs frequently in the dust of places inhabited by man, as well as on the surface of the skin and of the mucous membranes of the nose and mouth.

Diagnosis.—The staphylococcus pyogenes aureus cannot usually be identified with any certainty by the cover-glass examination alone. Cultures are necessary in order to differentiate from the other staphylococci and from the streptococcus.

For practical purposes the identification of the pyogenic cocci may be made by the appearances of their colonies on blood-serum and by their morphology; no secondary cultures are usually necessary.

The following staphylococci may also be present in acute inflammatory processes, but they occur less frequently than does the staphylococcus pyogenes aureus.

Staphylococcus Pyogenes Albus and Staphylococcus Pyogenes Citreus.—These organisms differ from the staphylococcus pyogenes aureus mainly in the color of their colonies. As a rule, they are much less pathogenic for rabbits than that organism.

Staphylococcus Epidermidis Albus (Welch).—"Is probably only a variety of the staphylococcus pyogenes albus. Usually grows somewhat more slowly; liquefies gelatin and coagulates milk less rapidly. Is of little virulence under ordinary conditions. Is a regular inhabitant of the epidermis, lying deeper than can be reached by disinfection of the surface of the skin" (Welch).

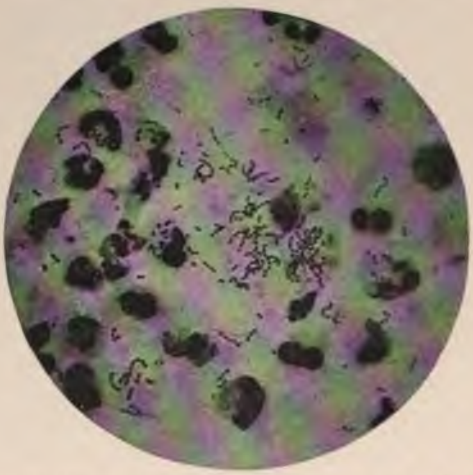

FIG. 32.—Streptococcus pyogenes; cover-glass preparation of the pus of an abscess; × 1000 (Fränkel and Pfeiffer).

Staphylococcus Cereus Albus.—Very similar to the staphylococcus pyogenes albus, but does not liquefy gelatin. May occur in abscesses.

Staphylococcus Cereus Flavus.—This organism is similar to the preceding, except that it forms a lemon-yellow pigment.

Streptococcus Pyogenes.[1]—This organism may be regarded as identical with the *streptococcus erysipelatos* of Fehleisen.[1]

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[1] Rosenbach; *loc. cit.*, p. 129.

Blood-serum.—Minute grayish-white colonies, often looking like small grains of sand scattered over the surface of the medium. Sometimes the colonies are shining, translucent, colorless, resembling minute dewdrops.

Morphology.—Rather small cocci arranged in chains, each coccus being divided into two hemispheres by a line of division running at right angles to the axis of the chain (Figs. 32, 33). The chains may be made up of many cocci and be quite long.

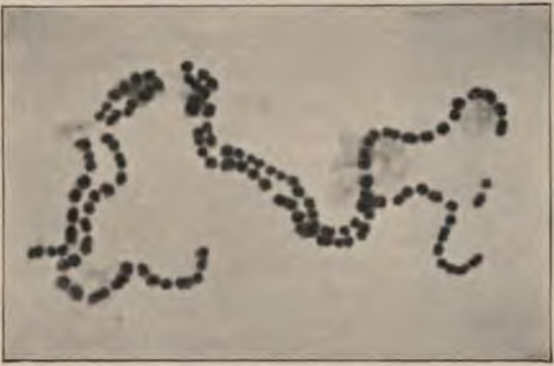

FIG. 33.—Streptococcus pyogenes from a culture in bouillon; × 2000 (Wright and Brown).

Cover-glass preparations from the colonies often fail to show the characteristic chain arrangement, owing to the chains being broken up by the manipulation. The chain-formation is best demonstrated in cover-glass preparations from the "water of condensation" at the bottom of the blood-serum tube. This is essentially a bouillon culture, and it is in such fluid media that the chain-formation is best developed. In preparing the cover-glass from this as little manipulation of the fluid as possible should be used, in order to avoid destroying the chain arrangement.

Practically, the only organism with which the streptococcus may be confounded is the pneumococcus, which also grows in minute colonies and sometimes in chains. The streptococcus may be distinguished from the pneumococcus, however, by the morphology of the individual organisms, the streptococci appearing as pairs of hemispheres, and the pneumococci as pairs of oval, conical, or lancet-shaped organisms, the broader ends of which are in apposition.

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[1] Fehleisen: *Die Aetiologie des Erysipels*, Berlin, 1883.

Stains by Gram's method.

Bouillon.—The character of the growth in bouillon is subject to considerable variation, and certain doubtful *varieties* of the streptococcus are distinguished mainly by the bouillon culture.

"We thus distinguish short-chained streptococci ('streptococcus brevis'), long-chained streptococci ('streptococcus longus'), streptococci which render bouillon cloudy and those which do not, streptococci which form flocculent or scaly or sandy or viscous sediments.

"The name 'streptococcus conglomeratus' is given to a streptococcus which grows, without clouding the bouillon, in the form of dense, separate particles, scales, or thin membranes at the bottom or sides of the tube, and on shaking the sediment it breaks up into little specks, without producing uniform diffuse cloudiness.

"On microscopical examination the chains in the latter case are long and interwoven in conglomerate masses. Streptococcus chains may be straight or wavy or twisted. These various distinctions are only of relative value. One form may change into another. Virulent streptococci may be found among all the groups mentioned; the streptococci of erysipelas and most of the streptococci from abscesses and septicemia grow in long chains in bouillon" (Welch).

Agar-agar Slant.—Minute grayish translucent colonies (Fig. 34).

Agar-agar Stab.—Small spherical grayish colonies along the needle-track.

Gelatin.—Growth similar to that on agar-agar.

Litmus-milk.—Some varieties turn the medium pink and cause coagulation.

Pathogenesis.—The results of the inoculation of animals are not constant, great variation in the virulence of different cultures being observed. Sometimes mice inoculated at the root of the tail or in the peritoneal cavity will die in about twenty-four hours with enlargement of the spleen and large numbers of the organism in the internal organs.

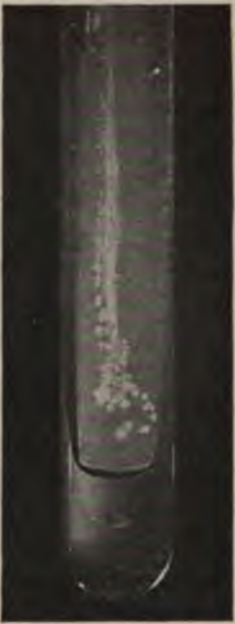

FIG. 34.—Streptococcus pyogenes: culture upon agar-agar two days old (Fränkel and Pfeiffer).

Occurrence.—The streptococcus occurs frequently in the spreading phlegmonous inflammations as well as in suppurative processes generally, and is the most common cause of septicemia. It is almost always present in inflammatory conditions of the mucous membrane of the pharynx, and is often encountered in broncho-pneumonia. In erysipelas it is almost invariably the infecting organism, and it is the most frequent cause of puerperal septicemia. In the majority of fatal cases of diphtheria and in some cases of typhoid fever, scarlet fever, tuberculosis, and other acute inflammatory diseases it will be found in the blood of the various internal organs after death. It also occurs in a certain proportion of cases of peritonitis, pleuritis, meningitis, endocarditis, and otitis media. Gaining entrance to the tissues through an insignificant wound or abrasion of the skin, it may produce a rapidly fatal septicemia in a susceptible individual, in whose internal organs at autopsy large numbers of the organism will be found. This general invasion of the circulation may also be observed in cases of chronic or wasting disease, the infection occurring during the last days or hours of life (terminal infection).

Of other conditions in which it may occur, hepatic abscess, appendicitis, osteomyelitis, and synovitis may be mentioned.

Although the streptococcus is distinctly one of the pus-producing bacteria, yet the inflammations of the soft parts of the extremities which are produced by it are generally characterized more by necrosis and serous or hemorrhagic exudation and infiltration than by the breaking down of tissue and frank pus-production. In this the organism is in marked contrast to the staphylococcus pyogenes aureus, which practically always produces dissolution of tissue and pus. Moreover, the streptococcus inflammations are more commonly accompanied by lymphangitis than are those due to the staphylococcus pyogenes aureus.

In a few instances we have met with a streptococcus whose colonies assume a well-marked yellow color on blood-serum, but which in other respects are like the long-chained forms above described.

Diagnosis.—The streptococcus pyogenes may often be identified by the cover-glass examination alone through its characteristic chain-formation, but this may not be apparent and the result of cultures must then be awaited.

Erysipelas.—The streptococcus is most readily found in the extreme margin of the affected area where the process is newest. The skin should be cleansed with soap and water, and with alcohol. Then with a sterile knife-point or a large needle a small wound should be made, and some of the blood and exudate pressed out from the tissue beneath. From this, cultures and cover-glasses may be prepared.

Pneumococcus.[1]—*Synonyms:* Diplococcus pneumoniæ; Micrococcus lanceolatus; Micrococcus of sputum-septicemia; Micrococcus pneumoniæ crouposæ.

Blood-serum.—Minute colorless, transparent colonies, resembling very small drops of dew.

Morphology.—Pairs of rather small oval, conical, or lancet-shaped organisms, the broader ends being in apposition. The organism varies somewhat in size, and one of the "pair" may be smaller than the other (Fig. 35). In some cases atypical or involution forms are seen, especially if the culture be more than twenty-four hours old. No capsules are ordinarily observed in cultures with ordinary methods of staining. In the "water of condensation" of the blood-serum tube, chains may be formed resembling those of the streptococcus, but differing from the chains of that organism by the oval or lancet form of the elements of which they are composed.

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[1] A. Fränkel: *Zeitschrift für klinische Medicin*, Bd. x. u. xi.; Weichselbaum: *Wiener med. Jahrbücher*, 1886.

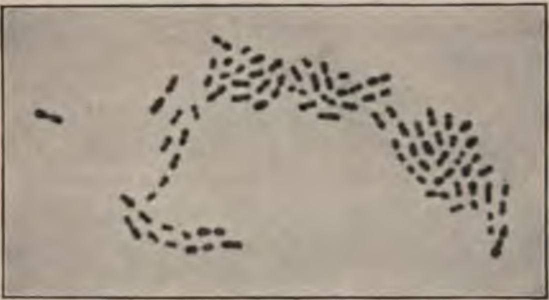

FIG. 35.—Pneumococci from a culture; × 2000 (Wright and Brown).

In pus, blood, or in other material, the organism is invested with a hyaline zone called the capsule (see Figs. 36, 37).

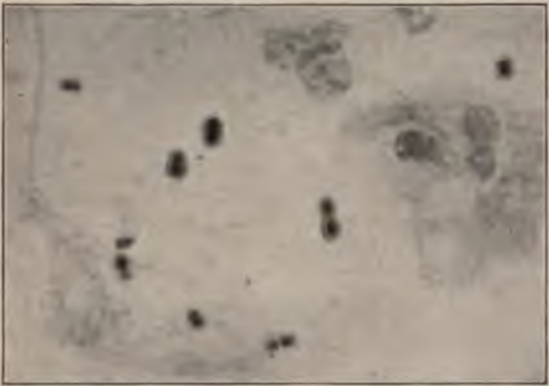

FIG. 36.—Pneumococci with capsules in a cover-glass preparation from sputum stained by Gram's method and Bismarck brown; × 2000 (Wright and Brown).

This is composed of a mucin-like substance. It may be seen usually in cover-glass preparations stained by the ordinary methods, especially if the preparations be examined in water-mounting.

Stained by Gram's method. Not motile.

Glycerin Agar-agar.—Feeble growth of very minute grayish colonies.

Bouillon.—Clouded faintly.

Litmus-milk.—Sometimes turned pink and coagulated. Growth on other culture-media is very feeble. The organism dies out rapidly in cultures. To keep it viable it should be transplanted every forty-eight hours.

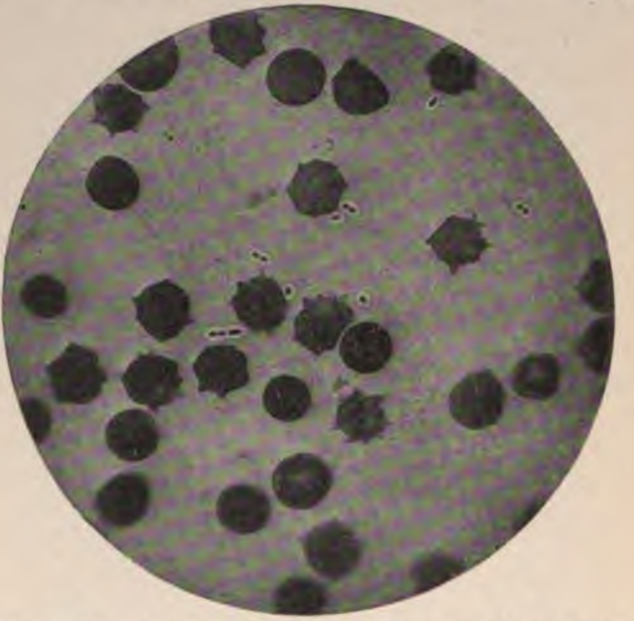

FIG. 37.—Diplococcus pneumoniæ; cover-glass preparation from the heart's blood of a rabbit; × 1000 (Fränkel and Pfeiffer).

Pathogenesis.—The pneumococcus is very pathogenic for mice and rabbits, less so for guinea-pigs.

Subcutaneous inoculation with virulent cultures causes the death of mice in from twenty-four to thirty-six hours, and of rabbits in from thirty-six to forty-eight hours, with septicemia.

This infection is the "sputum-septicemia" of Sternberg. At the autopsy there will be found in the blood everywhere the characteristic encapsulated lancet-shaped organisms, usually in pairs (Fig. 37). Great variation in the virulence of the organism is observed. In some cases no effect will be produced by the inoculation; in others a more or less extensive fibrino-purulent exudation will be produced about the point of inoculation, and the animal will survive for a considerable length of time or recover. Inoculation into the ear-vein or peritoneal cavity of a rabbit will sometimes cause a rapidly fatal septicemia, when subcutaneous inoculation with the same culture will only cause a local reaction. The virulence of the pneumococcus is quickly lessened by cultivation.

Occurrence.—The pneumococcus may be demonstrated in the pulmonary exudate of practically all cases of genuine lobar or croupous pneumonia. At autopsies on cases of this disease it may be found in large numbers in the consolidated lung, and sometimes in smaller numbers in the blood of other internal organs. Cultures from the lung may sometimes show the presence of other bacteria in addition to the pneumococcus, but these are to be regarded as either secondary infections or contaminations from the smaller bronchi.

The pneumococci in the pneumonic exudate die in large numbers after a time, and in cases near resolution numerous capsules may be found in cover-glass preparations from the lung in which it is impossible to demonstrate the organism by staining methods.

The pneumococcus is also frequently found in broncho-pneumonia, acute peri- and endo-carditis, acute pleuritis and empyema, acute purulent meningitis, and in otitis media. In cases of pneumonia and bronchitis it may be present in the sputum in large numbers. It has been observed in cases of peritonitis, of synovitis, of osteomyelitis, and of abscess-formation in various situations.

At autopsies on individuals dead of these conditions it may be frequently found, by means of cultures and animal inoculations, generally distributed throughout the internal organs in variable numbers. It is also often present in the mouth and in the saliva of healthy individuals.

Diagnosis.—If the pneumococcus be present in very small numbers in pathological material, the quickest and most certain method of demonstrating its presence is the inoculation of a mouse with some of the material (see page 116). This is also the best way to prove the identity of the organism.

The pneumococcus can usually be identified in exudates, blood, tissues, or sputum by examination of cover-glass preparations alone, by reason of its peculiar morphology and its possession of a capsule. The capsule can be seen in most instances in cover-glass preparations, stained in the usual manner, if they be examined in water-mount. The capsules appear as a hyaline material usually with definite outlines surrounding the paired organisms. If it be desired to stain the capsules the following methods may be used. Their working is somewhat uncertain.

Methods of Staining the Capsule of the Pneumococcus. —Welch's Method.—This method depends upon the fact that acetic acid precipitates the mucin-like substance of which the capsule is composed, and that the precipitated material of the capsule is not soluble in a 2 per cent. solution of sodium chlorid. Therefore, any necessary washing of the preparation is to be done in the solution of sodium chlorid, and the mount is to be made in the same solution or in balsam.

The method is as follows:

1. Cover the preparation with glacial acetic acid for a few seconds.

2. Drain off and replace (without washing in water) with aniline-gentian-violet solution. The staining solution is to be repeatedly added to the surface of the cover-glass until all of the acid is replaced.

3. Wash in a 2 per cent. solution of sodium chlorid and mount in the same.

W. H. Smith's Method.—This has been found particularly useful in demonstrating the pneumococcus in the sputum. The sputum or other material should be fresh. The cover-glasses should be spread as thinly as possible and fixed by passing three times through the flame in the usual manner.

1. Stain in aniline-gentian-violet solution for a few seconds, gently warming until the staining fluid steams.
2. Wash in water.
3. Cover with Gram's solution of iodin for thirty seconds.
4. Wash with 95 per cent. alcohol until the color ceases to come out.
5. Wash with ether for a few seconds. (To remove fat.)
6. Wash in absolute alcohol for a few seconds.
7. Stain one to two minutes in a saturated aqueous solution of eosin.
8. Wash with absolute alcohol for a few seconds.
9. Clear with xylol.
10. Mount in balsam.

The pneumococcus is stained blue-black while the capsule is stained pink. This method gives beautiful preparations. With the following modification it has been used by Smith as a routine stain for sputum. The advantage of this modification is that influenza bacilli and other bacteria which do not stain by Gram's method are clearly brought out, as are also eosinophilic leucocytes. This modification consists in washing the preparation with Löffler's alkaline methylene-blue solution just after it has been stained with eosin, as described above, and then, after the excess of eosin has been removed by the methylene-blue, steaming the methylene-blue solution for a few seconds while on the cover-glass. The preparation is then washed in water, rinsed with alcohol, cleared with xylol, and mounted in balsam.

Gonococcus.[1]—*Morphology.*—Cocci of medium size, composed usually of two hemispheres separated by a narrow unstained interval. Sometimes two of these pairs of hemispheres are joined together in the manner of "tetrads," or groups of four, showing evidence that division occurs in two directions at right angles to each other (Fig. 38). Decolorized by Gram's method.

The gonococcus will not grow upon any of the culture-media ordinarily employed, but requires special media for its cultivation.

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[1] Neisser: *Centralbl. f. d. med. Wissenschaften*, No. 28, 1879; Bumm: *Der Mikroorganismus der gonorrhoischen Schleimhauterkrankungen—"Gonococcus Neisser,"* Wiesbaden, 1887; Wertheim: *Archiv f. Gynäcologie*, Bd. 42, 1892.

The *colonies* on suitable culture-media appear after eighteen to twenty-four hours as minute, grayish, translucent points. Later they may attain a diameter of 2 mm. Under low magnifying power a well-developed colony is seen to consist of a generally circular expansion, with thin, translucent, sharply-defined margins, becoming brownish, granular, and denser toward the center, which is made up of coarse brownish clumps closely packed together.

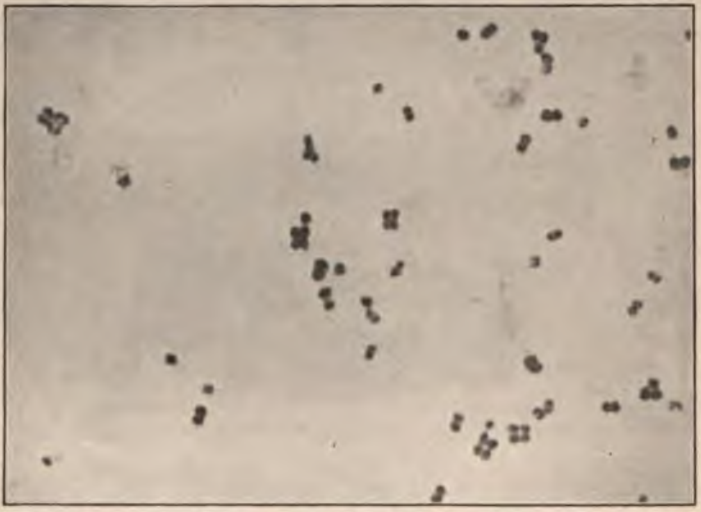

FIG. 38.—Gonococci from a culture, showing formation of tetrads and variability in the size of the cocci; × 2000 (Wright and Brown).

Special Culture-media.—The essential constituent of culture-media upon which the gonococcus will grow seems to be the blood-serum or similar albuminous fluid from the animal body.

Blood Agar.—Probably the most convenient means of cultivating the gonococcus is the use of blood agar as described for the cultivation of the bacillus of influenza (see p. 197).

Serum Agar-agar.—This medium consists of sterile human blood-serum mixed with fluid agar-agar at a temperature of

40° C. in the proportion of 1 part of blood-serum[1] to 2 or 3 parts of agar-agar. The blood-serum is to be obtained under the strictest precautions to avoid contamination with bacteria, thoroughly sterilized vessels, etc. being used. The blood may be conveniently drawn from the median vein of the arm with a sterilized antitoxin syringe. Placental blood obtained from the umbilical cord by pressure on the placenta may be also employed.

Ordinary tubes of plain agar-agar (2 per cent.) which have been previously sterilized in the usual manner are melted and brought to a temperature of 40° C. in a water-bath. To the fluid agar-agar in each tube the sterile blood-serum is then added in the proportion of one-third to one-half the volume of the agar-agar, care being taken to avoid contamination. For the transfer of the serum to the agar-agar tubes a sterilized pipette may be used. The tubes may then be infected and their contents poured into sterilized Petri dishes, as in the plate method previously described (see page 108), or the tubes may be placed on their sides in a slightly inclined position and the agar-agar allowed to solidify, thus forming "slants" which may be kept on hand ready for use. In order to test for the presence of contaminating bacteria in these slants, it is well to place them in the incubator for twenty-four hours after they have become solid, so that any bacteria which may be present in them will form colonies and manifest themselves. If only two or three tubes of this media are required, they may be prepared by drawing a suitable quantity of blood from the median vein of the arm with a sterilized hypodermic syringe, mixing this blood directly with tubes of melted agar-agar, as above indicated, and allowing the mixture to solidify to form "slants." A thorough cleaning of the skin about the vein is of course necessary. Some pathological fluids which are rich in albumin, such as the serous exudate of pleuritis, may be used in the place of blood-serum as above described. In using these, however, it is important to be sure that they contain albumin in considerable quantities, for they vary greatly in this respect, and those which contain but little albumin will probably be of no value for the purpose.

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[1] In place of the blood or blood-serum, hydrocele or pleuritic fluid, obtained under proper precautions to avoid contamination, may be used.

Urine-Serum-Agar-agar.—This medium is prepared by mixing, under aseptic precautions, with melted agar-agar at a temperature of about 40° C., one-third to one-half its volume of a sterile mixture of beef blood-serum and human urine.[1] The mixture of blood-serum and urine consists of 1 part urine and 2 parts serum. This mixture is freed from bacteria by filtration through a large unglazed porcelain filtering apparatus, and it is then added to the ordinary sterilized agar-agar (plain) tubes, which have been melted down and are kept fluid in a water-bath at about 40° C. After the admixture of the urine and serum with the agar-agar, the contents of the tubes are allowed to solidify in the form of "slants," and then tested for contaminations by placing the tubes in the incubator for twenty-four hours. The agar-agar, if used, should be more concentrated than usual to allow for the addition of the urine and serum. This concentration can best be obtained by evaporating the agar-agar before running it into the tubes to about three-fifths of its proper volume. Thus a liter of agar-agar may be evaporated by boiling to 600 or 700 c.c. The tubes should be filled with this concentrated agar-agar to a depth of about 3 cm. The filtering apparatus should be sterilized by steam before using, as also everything which comes in contact with the culture material. A half hour in the steam sterilizer will suffice for the purpose.

Wasserman's Culture-medium for Gonococci.—Mix in an Erlenmeyer flask 15 c.c. of pig's blood-serum, 30 to 35 c.c. of water, 2 or 3 c.c. of glycerin, and 0.8 to 0.9 gm. of nutrose. Shake the mixture, and heat it to boiling under continued shaking. The mixture clears up under heat, and may be sterilized by steaming without coagulation. It keeps indefinitely. This sterilized mixture is combined for culture purposes at 50° C. with equal parts of 2 per cent. nutrient melted agar.

The gonococcus is not pathogenic for laboratory animals, as is the case with certain other pathogenic bacteria. When inoculated with bits of agar into the eye or into the peritoneal cavity of mice and guinea-pigs it may give rise to suppuration.

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[1] J. H. Wright: "On the Cultivation of the Gonococcus from Cases of Gonorrhea, Ophthalmia Purulenta, and Pyosalpinx" (*Amer. Journal Med. Sci.*, Jan., 1895).

Occurrence.—The presence of the gonococcus may be demonstrated in the pus of acute gonorrhea and gonorrheal ophthalmia. It occurs also in a certain proportion of cases of purulent salpingitis. It has been found in peritonitis, endocarditis, pericarditis, myocarditis, pleuritis, and arthritis, as well as in periurethral abscess, in abscess of the glands of Bartolini, and in other acute inflammatory processes. In a few cases of endocarditis it has been demonstrated in the blood during life.

Diagnosis.—For practical purposes the gonococcus may be sufficiently identified in pus by cover-glass examination of the same. Cocci in the form of paired hemispheres chiefly situated within the pus-cells and decolorizing by Gram's method of staining may be regarded as gonococci with a fair degree of certainty. The fact that they decolorize by Gram's method serves to distinguish them from the pyogenic staphylococci and streptococci, for these may also be present inside leucocytes, and may in some instances look like gonococci. The identification by this means is not beyond question. To make it more certain the isolation and study of the suspected cocci in cultures are necessary. In cultures, not only should the organism show the peculiarities of morphology, of staining, and of colony growth, above described, but it should be rigidly determined that it does not grow on ordinary agar-agar.

If it is desired to obtain cultures of the gonococcus from the pus of gonorrheal urethritis, the case should not be more than of a few days' duration, because cases of longer duration will usually show the presence of other bacteria whose colonies overgrow the feebly growing colonies of the gonococcus. An organism which may be mistaken for the gonococcus is a coccus growing in large milk-white colonies on all media, but staining by Gram's method. This coccus is frequently found in gonorrheal pus after the discharge has lasted several days. Other cocci also occur.

The pus for culture purposes may be collected on a "swab," and the special culture-medium directly infected with this. The gonococcus retains its vitality in the pus on the swab for a number of hours, but care should be taken to avoid drying.

In a certain proportion of cases of purulent inflammation of the oviducts, gonococci may be found and cultivated, as above indicated. The majority of cases, however, will have sterile pus, while in a small percentage of cases the ordinary pyogenic cocci will be present.

Cultures from the blood in cases of gonorrheal endocarditis may give positive results. For the method of making such cultures, see page 98. The mixture of blood and agar, obtained by this method, forms a good medium for the growth of the gonococcus.

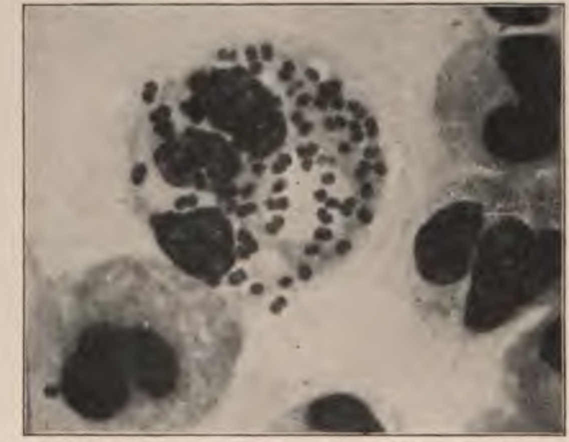

FIG. 39.—Gonococci inside a leucocyte. Cover-glass preparation from gonorrheal pus; × 2000 (Wright and Brown).

In applying the test of decolorization by Gram's method, colonies not more than forty-eight hours old should be used, because Gram-staining cocci in older cultures may be more or less decolorized by this method. In proof of the necessity of cultures for confirming the identity of the gonococcus in certain instances, we may state that we have met with a Gram-decolorizing coccus in an arthritis of the knee, clinically of gonorrheal origin, which, in cover-glass preparations from the exudate, was regarded as the gonococcus, but which was found not to be that organism by the study of it in cultures.

Method of Staining for Gonococci.—Prepare a cover-glass with the pus, spreading it thinly with the platinum wire. The practice of spreading a small drop of pus between two cover-glasses and drawing them apart is objectionable. After "fixing" stain the preparation by the following method:

1. Stain with aniline-gentian-violet solution for thirty seconds, without heating.
2. Wash in water.
3. Cover the preparation with Gram's iodin solution for thirty seconds.
4. Wash in water.
5. Wash with alcohol (95 per cent.) until the color ceases to come out of the preparation.
6. Wash in water.
7. Stain in saturated aqueous solution of Bismarck brown for thirty seconds.
8. Wash in water and mount.

This method is nothing but Gram's method and after-staining with Bismarck brown. With it the gonococci are stained brown, and other pyogenic cocci stained blue-black.

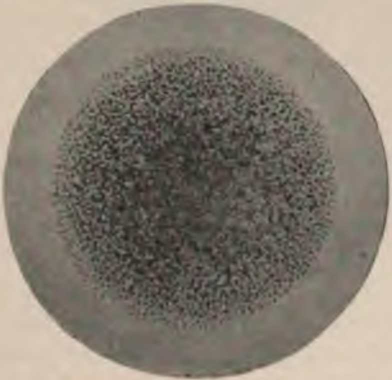

FIG. 40.—Micrococcus tetragenus: colony twenty-four hours old upon the surface of an agar-agar plate; × 100 (Heim).

Micrococcus Tetragenus.[1]—The *colonies* are small, white, and elevated (Fig. 40). Growth is slow.

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[1] Koch: *Mitth. a. d. Kais. Gesundheitsamte*, Bd. 2, 1884; Gaffky: *Arch. f. klin. Chirurgie*, Bd. 28.

Morphology.—Micrococci arranged in fours, or "tetrads," held together by a gelatinous substance (Fig. 41).

Stained by Gram's method. Not motile.

Gelatin Stab.—Feeble growth in the form of minute spherical masses along the line of stab with a small white slightly elevated point at the surface of the medium. The gelatin is not liquefied.

Agar-agar Slant.—Moist, glistening, grayish-white translucent streak with wavy margins.

Potato.—Growth is in the form of "a thick, irregular, slimy-looking patch."[1] The growth on agar-agar and on potato may be drawn into thin threads by the platinum wire.

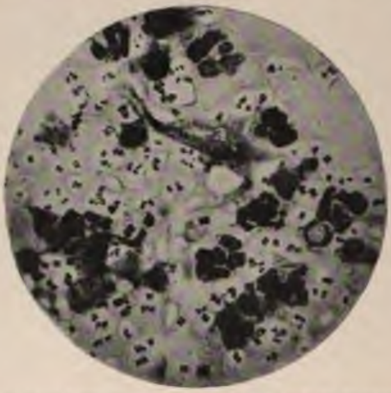

FIG. 41.—Micrococcus tetragenus in pus from a white mouse; × 615 (Heim).

Pathogenesis.—Subcutaneous inoculation of mice and guinea-pigs may lead to a fatal septicemia or only a local pus-formation. Intravenous or intraperitoneal inoculation of rabbits may also produce septicemia and death.

At autopsy the micrococci, arranged in tetrads, are found in the blood generally, but most numerously in the spleen. They can readily be demonstrated by cover-glass preparations.

Occurrence.—"Found not infrequently in phthisical cavities and sputum, occasionally in association with pyogenic cocci in abscesses connected with carious teeth and about the neck and jaws and middle ear, rarely in abscesses elsewhere. It has been considered to be non-pathogenic for man, but it has been found in pure culture in closed abscesses in man, and Viquerst has proven experimentally that it is capable of causing suppuration in human beings" (Welch).

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[1] Abbott: *Principles of Bacteriology.*

Diplococcus Intracellularis Meningitidis.[1]—*Morphology.*—Diplococci, occurring as paired hemispheres, separated by a well-marked unstained interval and showing considerable variation in size in cultures (Fig. 42). The larger forms are regarded as involution or degenerate forms. The organism shows a tendency to grouping in fours or tetrads.

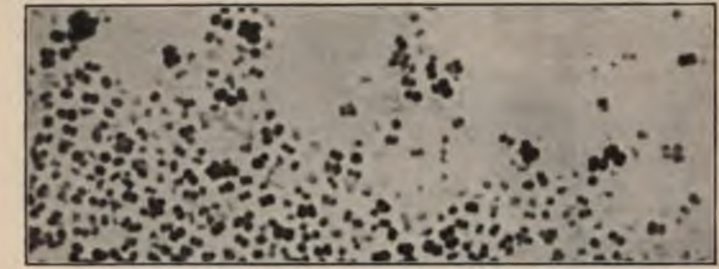

FIG. 42.—Diplococcus intracellularis meningitidis from a culture, showing the tendency toward grouping in fours or tetrads; × 2000 (Wright and Brown).

In cover-glass preparations from the meningeal exudate the diplococcus frequently is situated inside leucocytes, and sometimes within the nucleus (Fig. 43). The appearances are very much like those of gonorrheal pus. It is decolorized by Gram's method.

Blood-serum.—The colonies appear after about twenty-four hours, and after forty-eight hours may attain a diameter of 2 or 3 mm. They are round, colorless, shining, slightly convex or flat, moist, and viscid-looking. They may become confluent.

Agar-agar.—Round, flat, grayish, translucent, moist, shining colonies, attaining a diameter of 2 or 3 mm. after twenty-four hours in the incubator. They may become confluent, and in a "slant" culture the growth appears as a grayish, translucent, moist, shining streak about 3 mm. in width, with smooth margins. Under a low magnifying power the colonies are homogeneous, semi-translucent, and not granular.

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[1] Weichselbaum: *Fortschritte der Medicine*, Bd. 5, 1887; Jaeger: *Zeitschrift für Hygiene und Infectionskrankheiten*, Bd. 19, 1895; Councilman: *Transactions of the Association of American Physicians*, 1897.

Sugar-agar Stab-culture.—Feeble growth not extending all along the line of inoculation.

Bouillon.—Medium slightly to moderately clouded. At the bottom of the tube a whitish sediment, which may rise as a viscid string when the tube is shaken.

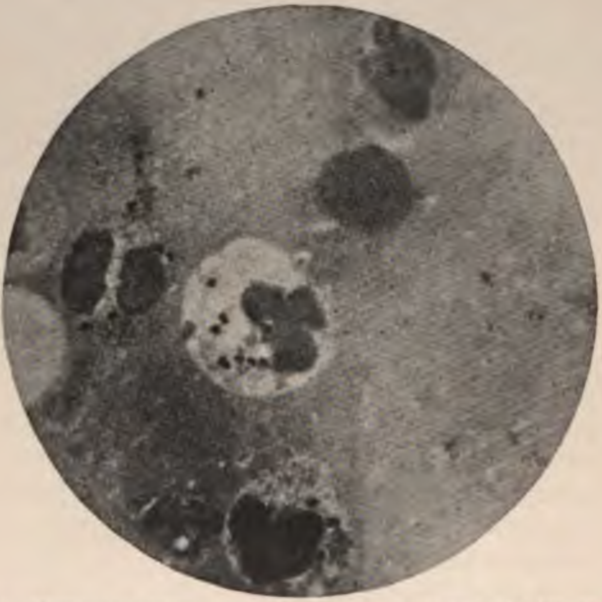

FIG. 43.—Diplococcus intracellularis meningitidis in polynuclear leucocytes of meningeal exudate (Jaeger).

Potato.—Very feeble or doubtful growth, giving the surface of the potato a moist appearance at the most.

Litmus-milk.—Growth without visible change in the medium.

Gelatin.—Feeble growth. No liquefaction.

Vitality.—The organism quickly dies out under cultivation. It seems to survive somewhat better on blood-serum than on agar-agar, but cultures on the former only seventy-two hours old may be found no longer capable of growth after transplantation.

Pathogenesis.—Intraperitoneal inoculation of guinea-pigs and rabbits gives very uncertain results. Mice are said to succumb to subcutaneous inoculation, with some invasion of the blood by the organism.

Exceptionally, we have found that the intraperitoneal injection of a bouillon suspension of a twenty-four-hour blood-serum culture in the quantity of about 1 c.c. would kill guinea-pigs within forty-eight hours.

At the autopsy there is an accumulation of a cloudy or blood-stained fluid in the peritoneal cavity, some little enlargement of the spleen, and some injection of the peritoneum. Microscopical examination of the exudate shows numerous leucocytes crowded with the diplococci (Fig. 44). The culture-test gives no evidence of general infection of the blood.

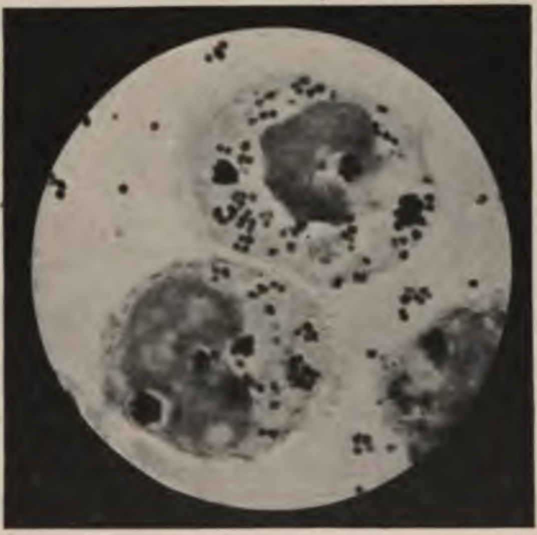

FIG. 44.—Diplococcus intracellularis meningitidis in leucocytes. Cover-glass preparation from peritoneal exudate in a guinea-pig; × 2000 (Wright and Brown).

Occurrence.—Found in the meningeal exudate of certain cases of epidemic cerebro-spinal meningitis. It is situated mainly inside the pus-cells, some of which may contain many diplococci. In some cases the presence of the organism in the exudate may be difficult or impossible to demonstrate, and it is probable that it rapidly dies out. It has been observed in the lung in the broncho-pneumonia of one case and in the nasal secretions of a number of cases. It has also been found in arthritis, which sometimes accompanies the disease. A general invasion of the circulation by the organism does not occur.

Diagnosis.—(See also the section on Lumbar Puncture).—In exudates suspected of containing this organism, cover-glass preparations should be stained by the method for gonococci (see page 147). The presence of brown-stained cocci, often in the pus-cells, is practically sufficient for the identification of the organism in cases of meningitis. In doubtful cases the study of cultures is necessary. It is claimed that the nasal secretion in the epidemic form of this disease contains the diplococcus. There is no doubt that diplococci, decolorizing by Gram's method, and often situated in polynuclear leucocytes, are to be found in the nasal secretion of certain cases of this disease, but whether their presence is pathognomonic of cerebro-spinal meningitis is not yet determined. The material for examination is best obtained with the platinum loop from the superior portions of the nasal cavities.

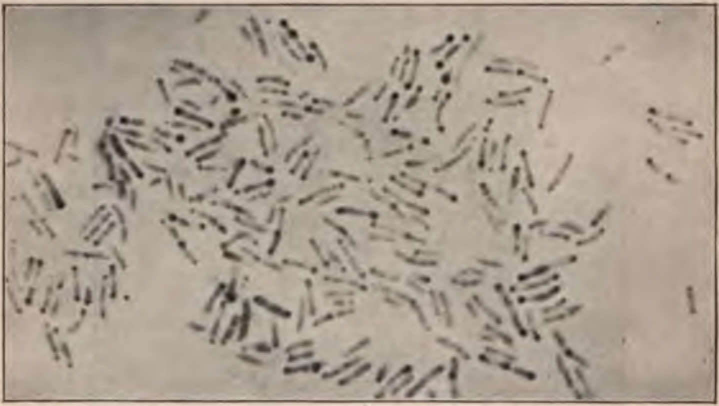

FIG. 45.—Diphtheria bacilli from a culture on blood-serum, stained by Löffler's methylene-blue solution, showing deeply stained points; × 2000 (Wright and Brown).

Bacillus Diphtheriæ.[1]—*Blood-serum.*—Round, elevated, smooth colonies of the color of the medium. They may attain a diameter of 2 mm. after forty-eight hours in the incubator.

Morphology.—Bacilli varying markedly in size and shape, of irregular outline, and showing great variability in the staining of different parts of their protoplasm (Figs. 45, 46). The presence in a palely tinted rod of deeply stained granules and points, frequently situated at the extremities, and the occurrence of irregular forms, often club-like in shape with a constriction in the middle, are appearances which are very characteristic of the bacillus when grown upon blood-serum and stained with Löffler's methylene-blue solution. Its morphology and staining peculiarities are so characteristic when cultivated upon blood-serum that the microscopical examination is in most cases sufficient for its identification.

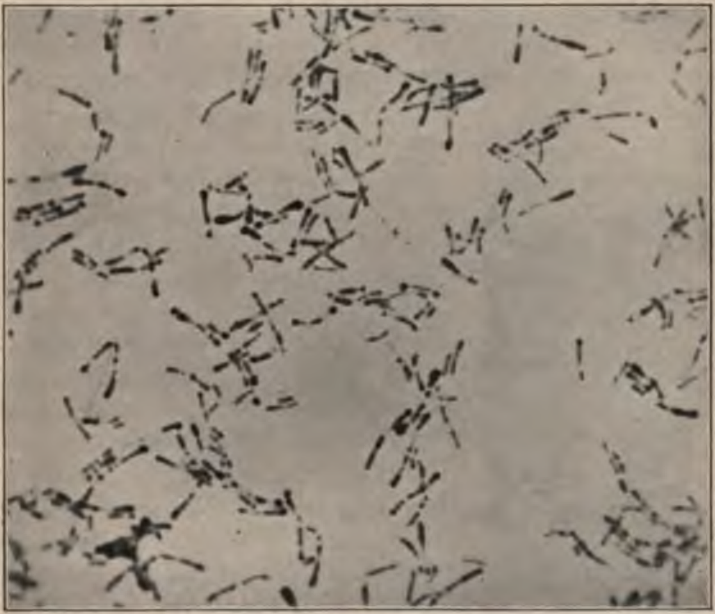

FIG. 46.—Diphtheria bacilli from a culture on blood-serum, stained by Löffler's methylene-blue solution, showing long and irregularly shaped forms of the bacillus, as well as the irregularity of staining; × 2000 (Wright and Brown).

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[1] Löffler: *Mitth. a. d. Kais. Gesundheitsamte*, Bd. 2, 1884; Roux and Yersin: *Annales de l'Institut Pasteur*, T. 2–4, 1888–90.

When grown upon other culture-media than blood-serum, however, its morphology and staining peculiarities are not so characteristic, and they may vary markedly in different media.

Stained by Gram's method. Not motile.

Bouillon.—Grows usually in the form of fine grains at the bottom of the tube and adherent to the sides, the bouillon remaining clear or becoming slightly clouded. The reaction of the media rapidly becomes acid, but changes to alkaline after a variable length of time.

Potato.—Growth not visible to the naked eye. The bacillus grows, however, to a certain extent, and usually assumes very atypical and irregular forms (involution forms).

Agar-agar and Gelatin.—The growth on these media is slower and more feeble than upon blood-serum (Fig. 47). It presents nothing remarkable.

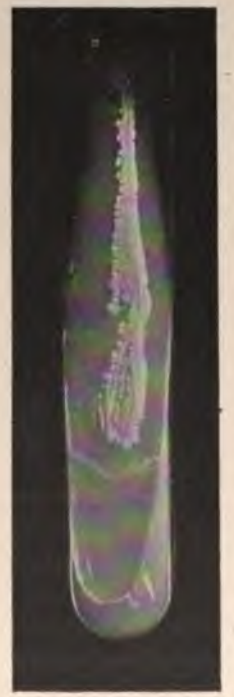

FIG. 47.—Bacillus diphtheriæ; agar-agar culture (photograph by Dr. Henry Koplik).

Pathogenesis.—Subcutaneous inoculations of guinea-pigs are fatal in thirty-six to seventy-two hours in the case of virulent cultures. The lesions produced consist usually of edema, hemorrhage, and fibrino-purulent exudation about the point of inoculation in the subcutaneous tissue, hemorrhagic enlargement of the lymphatic glands, congestion and edema of the lungs, hemorrhages into the suprarenal capsules, and less frequently necrosis of the liver and pleural effusions. Histological examination of the lymph-glands shows marked "fragmentation" of the nuclei of the cells, giving rise to numerous deeply staining globules of chromatin scattered throughout them. The bacilli are ordinarily found only about the point of inoculation, but cultures from the various organs will sometimes show the presence of the bacilli in some of them.

Toxin-production.—The effects produced by infection with the bacillus diphtheriæ are due to the action of a so-called toxalbumin or "toxin" which the organism manufactures in its growth. The poisonous substance is produced in cultures. Its presence may be demonstrated by inoculating an animal with a small quantity of the filtrate, obtained by passing a bouillon culture some weeks old through an unglazed porcelain filtering apparatus, by which all the bacteria are removed from the fluid.

The "toxin" is contained in solution in the filtrate. If this be fairly rich in "toxin," the injection of $\frac{1}{10}$ c.c. subcutaneously into a guinea-pig should lead to the death of the animal in three or four days with the various lesions above described. The local reaction, however, is not so marked as in the case of inoculation with the bacilli. With the ordinary bouillon the production of a great amount of "toxin" by the growth of the diphtheria bacilli in it is very uncertain. Theobald Smith has recently shown that this uncertainty is due to the presence of variable amounts of muscle-sugar from the meat used in the preparation of the bouillon, and that this substance prevents the accumulation of toxin. He has found that that bouillon yields the most toxin which has the least muscle-sugar in it. He prepares such bouillon as follows: "Beef infusion, prepared either by extracting in the cold or at 60° C., is inoculated in the evening with a rich fluid culture of some acid-producing bacterium (I use temporarily B. coli) and placed in the thermostat. Early next morning the infusion, covered with a thin layer of broth, is boiled, filtered, pepton and salt added, and the neutralization and sterilization carried on as usual." This bouillon is placed in two 500 c.c. Erlenmeyer flasks, 250 c.c. in each flask. In these, cultures are made and kept for at least eight days in the incubator. After this time a fair amount of toxin may be assumed to have developed, and the contents of the flask are then filtered through a porcelain cylinder. A filtrate is to be regarded as containing a reasonable amount of toxin if $\frac{1}{10}$ c.c., injected subcutaneously, kills a medium-sized guinea-pig in three days. The filtrate containing the "toxin" can be preserved by the addition of 0.5 per cent. pure carbolic acid.

Occurrence.—The bacillus diphtheriæ occurs in the local lesions in all cases of true diphtheria, in rhinitis fibrinosa, and in many cases of the milder forms of acute inflammation of the air-passages. It may persist in the mucous membrane of the throat and nose long after convalescence has been established.

In fatal cases of diphtheria the organism is nearly always present in the lungs, and it may be often found by culture-methods more or less generally distributed in comparatively small numbers throughout the internal organs. In the majority of diphtheria autopsies an invasion of the blood-stream by the streptococcus pyogenes, and sometimes by other bacteria, may be demonstrated by cultures. The bacillus may also be found in company with other bacteria in ulcerated or excoriated surfaces on the skin, as well as in other suppurative processes, in individuals affected with diphtheria, and on the soiled linen of the patient. The infection of wounds with the bacillus diphtheriæ has also been observed without coincident diphtheria.

Diagnosis.—The bacteriological diagnosis of infection with the bacillus diphtheriæ depends upon the characteristic morphology and peculiarities of staining, as well as rapidity of growth, which this organism presents when cultivated upon coagulated blood-serum. The identification by direct cover-glass examination of the exudate is very uncertain.

The method is as follows: A blood-serum culture-tube is inoculated with a small amount of the material from the mucous membrane affected, and is placed in the incubator twelve to eighteen hours. After this length of time the resulting growth is examined by cover-glass preparations stained either with Löffler's methylene-blue solution or by one of the special methods given below.

The bacillus diphtheriæ, if present, may then be recognized and differentiated from other bacteria present in the preparation by its characteristic morphology and peculiarity of staining, described on page 153. The gross appearances of the culture present little that is characteristic, as a rule, and the main reliance is to be placed on the microscopic examination. Early in the infection the greater part of the growth may be made up of the specific bacilli, but toward convalescence they fall into the minority. The ordinary forms of agar-agar culture are not suitable for use in the bacteriological diagnosis of diphtheria, owing to the comparative feebleness of the growth of the organism on these media, and because of the fact that its microscopic appearances when cultivated on such media are not sufficiently characteristic.

The material for culture is very conveniently obtained by means of sterilized cotton swabs. In collecting this material the swab is removed from its test-tube and touched to the affected areas of the mucous membrane of either the nose or throat. It is then to be gently rubbed over the surface of a blood-serum culture-tube, or it may be replaced in the test-tube and the inoculation of the culture-tube made later in the laboratory. In the latter case the inoculation should be made within an hour or two after the material has been collected, the infected swab meanwhile being prevented from drying by firmly replacing the cotton plug.

In cases with membrane-formation the greatest numbe- of bacilli are on the surface or in the upper layer of the membrane, and the swab should therefore be touched to these portions rather than to the tissue beneath.

Special Methods of Staining the Bacillus Diphtheriæ.—Owing to the fact that the bacillus diphtheriæ may be recognized by its peculiar morphology and characteristic staining in cover-glass preparations from its growth upon certain culture-media, as already pointed out, various special staining methods have been devised for accentuating and rendering more striking to the eye the peculiar deeply stained points and granules in the bodies of the individual bacilli, which have been referred to as of great importance in the identification of the organism.

These special methods of staining are said to be of great advantage in cases where only a few specific bacilli may be suspected to be present among a large number of other bacteria.

Neisser's Method.—1. Stain for one to three seconds in a solution which is made as follows: 1 gram of methylene-blue (Gruebler), in powder, is dissolved in 20 c.c. of 96 per cent. alcohol. To this add 950 c.c. of distilled water and 50 c.c. of glacial acetic acid, and filter.

2. Wash in water.

3. Stain for three to five seconds in a solution of vesuvin (Bismarck brown), made by dissolving 2 grams of the dye (in powder) in 1000 c.c. of boiling distilled water.

4. Wash in water, and mount.

The diphtheria bacilli stained by this method appear as pale brown rods bearing bluish-black granules, usually of oval shape and of a diameter somewhat greater than the rod. The majority of the bacilli show a granule at each end or at only one end, but not rarely three granules are present, one being near the middle of the rod. More granules than these are exceptional (see Fig. 48).

The bacilli must have been grown on Löffler's blood-serum medium, coagulated at 100° C., and the culture must be at least nine hours and not more than twenty-four hours old.

Hunt's Method.—1. Stain in saturated aqueous solution of methylene-blue one minute without heating.

2. Wash in water.

3. Cover with aqueous solution of tannic acid, 10 per cent., for ten seconds.

4. Wash in water.

5. Stain in saturated aqueous solution of methyl-orange one minute, without heating.

6. Wash in water.

7. Dry, and mount in balsam.

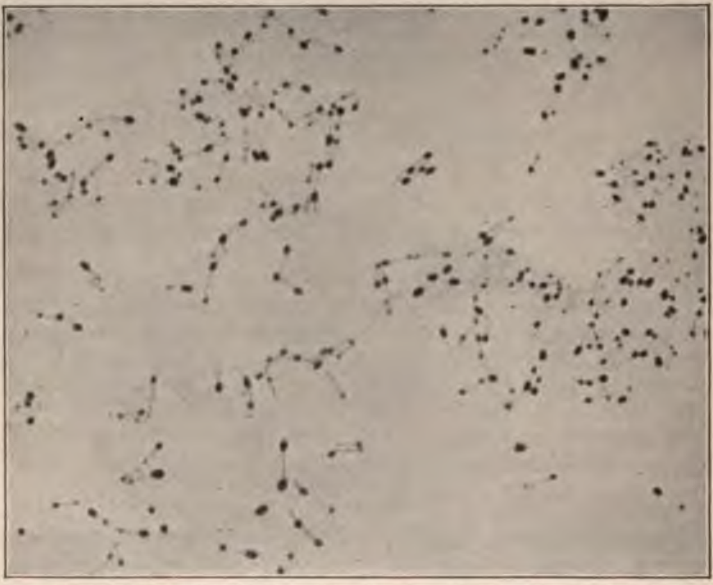

FIG. 48.—Diphtheria bacilli from blood-serum culture stained according to Neisser's method; × 2000 (Wright and Brown).

By this method the granules, etc., are dark blue or almost black, and stand out very sharply against the light-green coloring of the body of the bacillus (see Fig. 49). The solution of methyl-orange should be freshly prepared, for it deteriorates in a few days.

Bacillus of Typhoid Fever.[1]—*Synonyms:* Bacillus typhi abdominalis; Bacillus typhosus; Typhoid bacillus (see also Clinical Bacteriology).

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[1] Eberth: *Virchow's Arch. f. Path. Anat.*, Bd. 81, 1880; Bd. 83, 1881; Gaffky: *Mitth. a. d. Kais. Gesundheitsamte*, Bd. 2, 1884.

Blood-serum.—Round, grayish, viscid-looking colonies, which may attain a diameter of 2 mm. after forty-eight hours in the incubator.

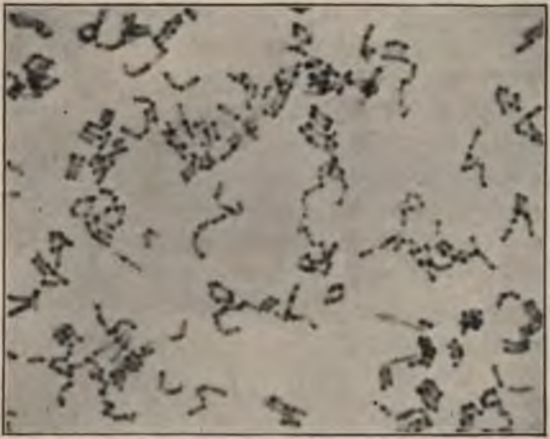

FIG. 49.—Diphtheria bacilli from blood-serum culture stained according to Hunt's method; × 2000 (Wright and Brown).

Morphology.—Medium-sized bacilli with rounded ends, generally short (Fig. 50), but sometimes long or thread-like, and frequently showing faintly-stained, sharply-defined areas in their protoplasm (Figs. 50 and 51).

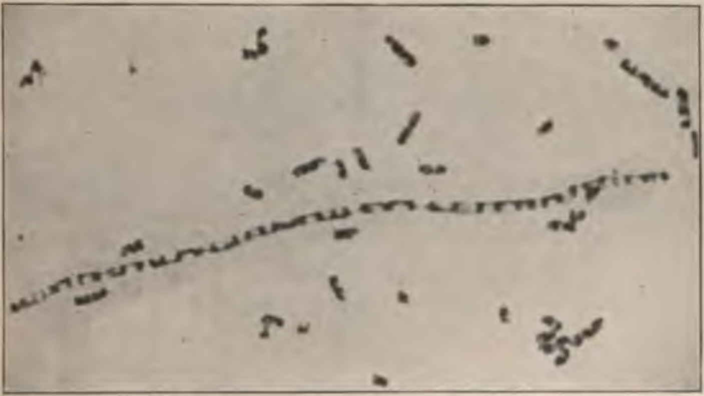

FIG. 50.—Typhoid bacilli from a bouillon culture, showing characteristic irregularity in staining and variability in length; × 2000 (Wright and Brown).

Gelatin Slant.—Broad translucent streak with wavy, irregular margins. The gelatin is not liquefied. Growth is slower than that of the bacillus coli communis in the same medium.

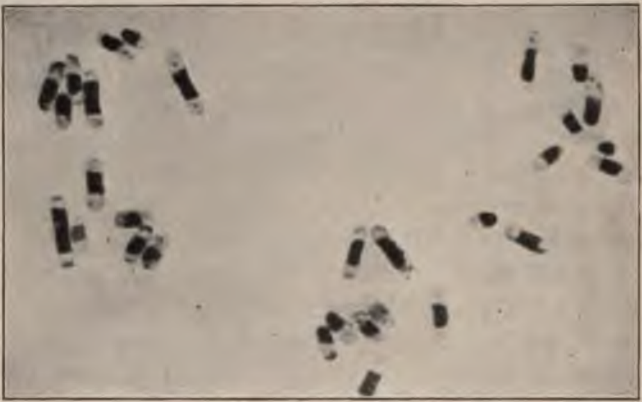

FIG. 51.—Typhoid bacilli from a culture on potato, showing unstained areas in the bacilli and polar granules; × 2000 (Wright and Brown).

An isolated colony, slightly magnified, on gelatin, is shown in Fig. 52.

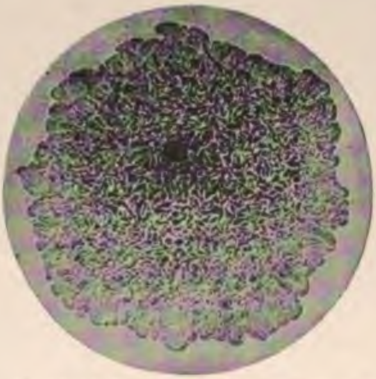

FIG. 52.—Bacillus of typhoid fever: superficial colony two days old, as seen upon the surface of a gelatin plate; × 20 (Heim).

Glucose-gelatin Stab.—Growth all along the line of inoculation in the form of confluent spherical grayish colonies, and on the surface about the point of entrance of the platinum wire in the form of a circular translucent grayish layer. No production of gas-bubbles. No liquefaction.

Glucose Agar-agar.—Growth similar to that in the preceding. No gas-formation.

Litmus-milk.—No visible change.

Potato.—Growth occurs, but it is usually invisible.

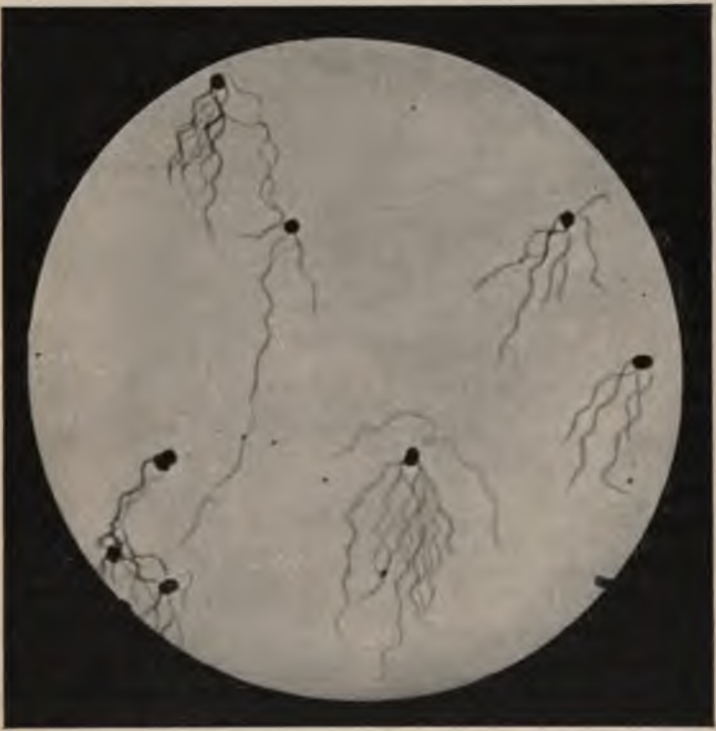

FIG. 53.—Typhoid bacilli, from a culture on agar-agar, showing flagella, from a preparation stained by Dr. Hugh Williams; × 2000 (Wright and Brown).

Dunham's Pepton Solution.—No indol-production—*i. e.* no red color appearing in the twenty-four- to forty-eight-hour cultures after the addition of 5 drops of concentrated sulphuric acid, c. p., and 1 cubic centimeter of a solution of sodium nitrite, 1 : 10,000.

Motility.—Very marked.

Flagella (Fig. 53) may be demonstrated by the special methods of staining described elsewhere.

Decolorized by Gram's method. Does not form spores.

Bouillon.—Clouded, with the formation of some sediment. The clouding of the medium is not so marked as in the case of the bacillus coli communis. In general, the growth of the typhoid organism is not so vigorous on culture-media as is the growth of the bacillus coli communis.

When to a bouillon culture a small quantity of the blood-serum of a typhoid-fever patient is added, the bacilli lose their motility and aggregate in clumps ("serum reaction").

Pathogenesis.—The inoculation of animals is usually without results if moderate quantities of the organism are used. Sometimes, however, death occurs apparently from the effects of the toxic material injected.

Occurrence.—Found in the spleen in large numbers at autopsies in typhoid fever. Its presence may also be demonstrated in the intestinal lesions, rose spots, mesenteric lymph-glands, liver, bile, kidneys, urine, and blood of the heart. As a rule, the number of bacilli found in the liver, kidneys, and blood of the heart is small. In the bile they may be numerous and may persist in it for a long period of time after the disease has subsided. In some cases the urine contains enormous numbers of the bacilli.

The typhoid bacillus may also occur in the suppurative sequelæ of typhoid fever, especially those involving bones. In these conditions, however, it may be accompanied by the pyogenic cocci. Occurs in contaminated water.

Differential Diagnosis between the Bacillus of Typhoid Fever and the Bacillus Coli Communis.—The most important points of difference between these two organisms are as follows, and to distinguish with certainty between them it is necessary that attention be paid to all of them:

Motility.—The typhoid bacillus is actively motile, the bacillus coli communis not motile or exceptionally motile.

Potato Cultures.—The typhoid bacillus usually grows invisibly, the bacillus coli communis as a dirty, slimy layer.

Gas-production in Media containing Glucose.—The bacillus coli communis produces gas, the typhoid bacillus does not.

Litmus-milk Cultures.—The bacillus coli communis changes the blue color of the medium to a pink color and usually coagulates the milk. The typhoid bacillus produces no visible change.

Indol-production.—The bacillus coli communis produces indol, the typhoid bacillus does not.

Serum or Clump Reaction.—The typhoid bacillus shows the clump reaction, while the bacillus coli communis does not. As it is not always possible to have a typhoid serum at hand by which to determine whether this reaction is present, a stock of dried blood from a typhoid case, contained in filter-paper, may be kept ready for use. That this is quite practicable has been recently shown by Dr. Mark W. Richardson. The blood may be obtained from the heart at the autopsy of a typhoid-fever case by soaking a piece of filter-paper with it. This is allowed to dry, and then is cut into pieces about 1 cm. square. When it is desired to make the test, one of these pieces is extracted with ten or fifteen drops of water, and a drop of this extract is mixed with a drop of an eighteen- to twenty-four-hour bouillon culture on a slide, covered with a cover-glass, and examined with the high-power dry lens. Dr. Richardson has found that the blood under these conditions retains for months its "clumping" power with reference to the typhoid bacillus.

Other differences are—the production of a red color in litmus-lactose agar-agar by the bacillus coli communis, and no change in color of this medium by the typhoid bacillus, and the slower and less vigorous growth of the typhoid bacillus in culture-media.

The Blood-serum Reaction in Typhoid Fever.—A few drops of the blood of a suspected case of typhoid fever are collected in a small test-tube, either from the finger or the ear. After clotting has taken place, transfer a drop of the serum by means of a medicine-dropper to 10 drops of a recent bouillon culture of the typhoid bacillus. After mixing, place a drop of the mixture on a slide, cover it with a cover-glass, and examine it with either an oil-immersion lens or a high-power dry lens. If desired, the mixture may also be examined as a hanging-drop preparation.

The preparation should be observed for at least twenty minutes. If clumping has not occurred in this time, the reaction is said to be absent. This is the procedure employed in the laboratory of the Massachusetts General Hospital. If the patient has typhoid fever, the majority of the bacilli on the preparation will be seen to lose their motility and to agglomerate into clumps within a very few minutes. This constitutes the serum-reaction. It is only demonstrable, as a rule, after the first week of the disease. The bouillon culture used should be grown at room-temperature and should not be more than four or five days old. Before carrying out the test it is well to assure one's self that the bacteria are actively motile by examining a drop of the culture as above indicated. The stock cultures of the typhoid bacilli are best made on agar-agar.

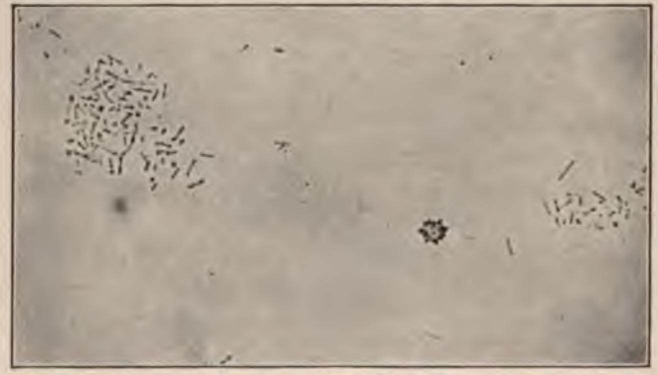

FIG. 54.—Showing the clumping of typhoid bacilli in the serum-reaction. Wet preparation, not stained. At one point a crenated red blood-corpuscle is seen (Wright and Brown).

The reaction may also be obtained from the dried blood. A few drops of the blood may be collected on a glass slide or a piece of paper and allowed to dry. It may then be brought to the laboratory, where as much of the dried blood as would correspond to a drop is scraped from the slide into a small test-tube, containing ten drops of a bouillon-culture of the typhoid bacillus, or as much of the paper as may be assumed to contain one drop of blood is placed in ten drops of the bouillon culture and allowed to soak for a few minutes therein. Microscopical examination is then made with the mixture thus obtained as above indicated.

Cultivation of the Typhoid Bacillus from Rose Spots.—Dr. Mark W. Richardson, of Boston, uses the following method: The skin is first washed with corrosive sublimate and then with alcohol and ether. The skin about the spot is then frozen with ethyl chlorid and a small crucial incision made in it. The wound is immediately scraped with a very small curette, and with the material so obtained a bouillon tube is inoculated. It is not to be expected that the bacillus will develop in every culture so made.

Cultivation of the Typhoid Bacillus from the Feces.—Piorkowski has devised the following culture-medium for this purpose: To a quantity of urine of a specific gravity of 1020, which has acquired an alkaline reaction by standing, 0.5 per cent. of peptone and 3.3 per cent. of gelatin are added. This mixture is heated over a water-bath for three-quarters of an hour, and immediately filtered and distributed in test-tubes in the usual manner.

The medium in the tube should be sterilized by steam only twice, fifteen minutes on the first day, and ten minutes on the second day.

In plate-cultures from the feces made with this medium the colonies of the typhoid bacillus appear in very irregular shapes provided with filamentous processes, and thus may be distinguished from the colonies of other bacteria.

Bacillus Coli Communis.[1]—*Synonyms:* Bacterium coli commune; Colon bacillus.

Blood-serum.—Rounded, grayish-white, slightly elevated, viscid-looking colonies, which may attain a diameter of 3 mm. after twenty-four hours in the incubator.

Morphology.—A medium-sized bacillus with rounded ends, often short or even coccus-like, but may grow in long forms. Faintly staining, sharply defined areas are present in the protoplasm (Fig. 55).

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[1] Escherich: *Fortschr. d. Medicin*, 1885, Nos. 16, 17.

Gelatin Slant.—Grayish translucent strip with wavy margins. The gelatin is not liquefied. Growth is more rapid than in the case of the typhoid bacillus.

A single colony on a gelatin plate is shown in Fig. 56.

Glucose-gelatin Stab.—Growth along the line of stab in the form of confluent spherical colonies, and on the surface about the point of entrance of the needle as a thin gray circular layer. Gas-bubbles are produced in the gelatin from fermentation of the glucose. The gelatin is not liquefied.

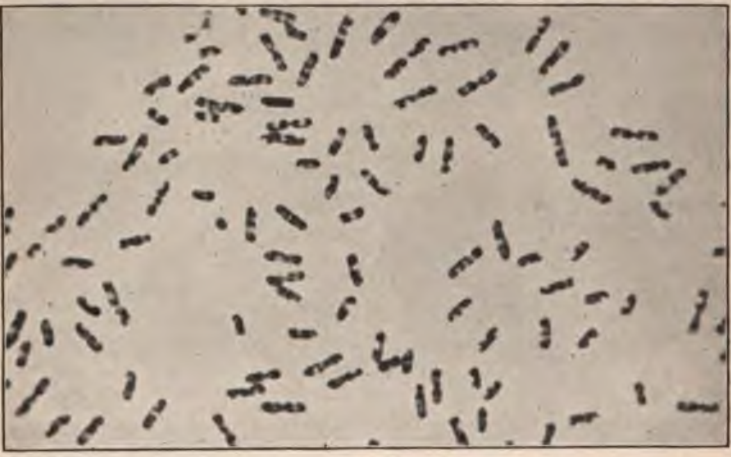

FIG. 55.—Bacilli coli communis from a bouillon culture, showing the irregularity of staining of the bacillus; × 2000 (Wright and Brown).

Glucose-agar-agar Stab.—Growth essentially the same as in the preceding, except that the gas-formation is more marked.

Litmus-milk.—Turned pink and usually coagulated.

Potato.—Dirty grayish or brownish, viscid-looking layer.

Dunham's Pepton Solution.—Marked indol-production. This is shown by the appearance of a red color in the culture after the addition of 5 drops of concentrated sulphuric acid, c. p., and 1 c.c. of a 1 : 10,000 solution of sodium nitrite. The culture in the pepton solution should have been at least twenty-four hours—or, better, forty-eight hours—in the incubator before the test is made.

Motility.—Usually not motile, but some varieties show sluggish independent movement.

Flagella.—May be demonstrated by the special methods of staining. They are less numerous than in the case of the typhoid bacillus.

Decolorized by Gram's method.

Bouillon.—Markedly clouded with formation of a sediment. The clouding is more marked than in the case of the typhoid bacillus.

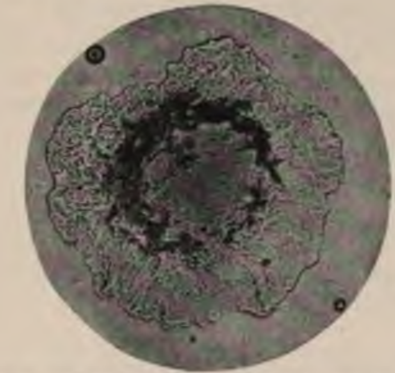

FIG. 56.—Bacillus coli communis: superficial colony two days old upon a gelatin plate; × 21 (Heim).

Lactose-litmus-agar-agar Slant.—Growth has a pink color, and the blue color of the medium is changed to red.

Pathogenesis.—"Its virulence as tested upon animals is variable, but is generally manifest only after inoculation of large doses, which kill by intoxication rather than infection" (Welch).

The lesions produced are not sufficiently characteristic to be detailed here.

Occurrence.—Occurs constantly in the intestinal canal, and is widely distributed in the external world.

"The colon bacillus is a frequent invader of the internal organs in all sorts of diseases, especially when there are intestinal lesions. It manifests no evident pathogenic action in most of these cases, and is then without clinical significance. It occurs frequently associated with other bacteria in infected wounds and other inflammations of exposed surfaces. Here also it does not usually appear to cause serious disturbance. The fact that the colon bacillus is so common and widely distributed, and found so often as a harmless invader, should lead to much caution in interpreting the significance of its presence when it occurs in definite lesions. There is no doubt, however, that it may be pathogenic for man. It plays an important rôle in inflammations of the urinary tract and biliary passages; also, but usually with less independence, in peritonitis and appendicitis.

"The list of diseases in which it may be found is a very long one and includes inflammations in all organs and parts of the body. In general its pathogenic properties are of a mild character. One of its leading rôles is to invade territory already occupied by other bacteria or previously damaged. It may be concerned in the production of gall-stones, in the interior of which it has been found by the writer with great frequency" (Welch, Dennis's *System of Surgery*, vol. i.).

The bacillus above described is to be regarded as a type of a group of bacilli constituting the so-called "colon group" of bacilli. These present certain quantitative differences among themselves which are not quite sufficient to characterize them as distinct species.

Welch regards as belonging to this group the *Bacillus pyogenes fœtidus*, distinguished by the stinking odor of its cultures, and the *Bacillus lactis aërogenes*, which is characterized chiefly by its plumper form, its more energetic gas-production, its rapid coagulation of milk, and its denser growth in cultures.

Theobald Smith[1] suggests that only those forms may be regarded as typical members of the group which grow on gelatin in the form of delicate bluish or more opaque whitish expansions with irregular margin, which are actively motile when examined in the hanging-drop from young surface-colonies taken from gelatin plates, which coagulate milk within a few days; grow upon potato either as a rich-pale or brownish-yellow deposit, or merely as a glistening, barely recognizable layer, and which give a dis-

[1] *Amer. Jour. Med. Sci.*, Sept., 1895.

tinct indol-reaction. Their behavior in the fermentation-tube[1] must conform to the following scheme:

[1] The fermentation-tube is a special form of culture-tube which may be obtained from dealers in bacteriological supplies. The closed branch of the tube should be completely filled with culture-fluid, but no more fluid should be placed in the tube than can be conveniently held by the open branch of the tube, so that if gas be formed in the closed branch the culture-fluid will not be forced out of the apparatus. The bubbles which collect at the top of the closed branch, after heating during sterilization, should be removed by an appropriate tilting of the tube. Theobald Smith,* who was the first to demonstrate the great value of the fermentation-tube in bacteriology, thus describes the mode of its use:

"The tubes are kept, after inoculation, in the thermostat at 37° C. A mark made on the sides of the closed branch at the end of every twenty-four hours with a glass pencil furnishes an approximate record of the rate of gas-production. Unless this is done it is impossible to know precisely when the formation of gas is at an end, and also whether or not the volume of gas has been diminished by absorption. It is best to wait four or five days after the production has ceased before making a final examination. This is done by noting the condition of the growth, the reaction of the fluid in the bulb,† and the maximum quantity of gas produced. This is most easily done by laying directly on the tube a glass millimeter rule, and noting the tube length occupied by gas. The entire length of the closed branch is also noted, making due allowance for the upper convex extremity and the lower constriction. This mode of measurement is sufficient, since only comparative values are desired. For the same reason all barometric and thermometric corrections are omitted in these approximate estimations.

"The examination of the gas produced was limited to the determination of the quantity of carbon dioxid and of the explosive character of the gas remaining after the absorption of CO_2 by sodium hydrate. These facts are determined by the following simple manipulations:

"The bulb is completely filled with a 2 per cent. solution of NaHO, and closed tightly with the thumb. The fluid is shaken thoroughly with the gas, and allowed to flow back and forth from bulb to closed branch and the reverse several times, to insure intimate contact of the CO_2 with the alkali. Lastly, *before removing the thumb, all the gas is allowed to collect in the closed branch* so that none may escape when the thumb is removed. If CO_2 was present, a partial vacuum in the closed branch causes the fluid to rise suddenly when the thumb is removed. After allowing the layer of foam to subside somewhat, the glass scale is again applied to the closed branch, and the amount of CO_2 absorbed may thus be measured. In all cultures of this character thus far examined the gas remaining was explosive in character, and probably hydrogen. The explosive character of this residue is easily demonstrated as follows: The wn).

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* *The Wilder Quarter-Century Book*, Ithaca, 1893, pp. 186, 187.

† The reaction was noted by placing a drop of the fluid on delicate litmus paper. The cultures were occasionally boiled to drive off any CO_2. In no case did the reaction with the litmus paper change.

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Variety *α*. One per cent. dextrose-bouillon (at 37° C.).

Total gas, approximately 1/2; H/CO_2 approximately 2/1; reaction strongly acid.

One per cent. lactose-bouillon:

As in dextrose-bouillon (with slight variation).

One per cent. saccharose-bouillon:

Gas-production slower than in the preceding, lasting from seven to fourteen days. Total gas finally about 2/3; H/CO_2 nearly 3/2. The final reaction in the bulb may be slightly acid or alkaline, according to the rate of gas-production.

Variety *β*. The same in all respects excepting as to its behavior in saccharose-bouillon. Neither gas nor acids are formed in it.

The Detection of the Bacillus Coli Communis in Water.—The presence of bacilli of the "colon group" in water is to be regarded as good evidence of pollution with fecal material from man or animals.

Theobald Smith's Method.[1]—A certain quantity of the water is added "to every one of ten fermentation-tubes containing a 1 per cent. dextrose-bouillon. This is added most easily by first diluting the water, so that 1 or 2 c.cm. are equivalent to the quantity which it is desired to add to each tube. Pipettes graduated by drops are convenient, but not so accurate." "When gas begins to appear in the fermentation-tubes the amount accumulated at the end of each twenty-four hours should be marked with a glass pencil on the tube. From these tubes, which contain 50 to 60 per cent. of gas on the third day, and are very strongly acid, plates may be prepared to confirm the indications of *B. coli*. This, however, is not essential, for the writer has found as yet no species having these fermentative characters which is not one of the following: *B. coli*, *B. lactis aërogenes*, *B. enteritidis*, *B. typhi murium*, *B. choleræ suis*. The three last-mentioned species are probably as rare in water as *B. typhosus* itself."

Bacillus Tuberculosis.[2]—*Synonyms:* Tubercle bacillus; Bacillus of Koch.

Blood-serum.—After three or four weeks in the incubator the colonies appear as dry, cream-colored, granular, slightly elevated patches with irregular margins, 1 to 2 mm. in diam-

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cotton plug is replaced, and the gas in the closed branch allowed to flow into the bulb, and mix with the air there present. The plug is then removed, and a lighted match inserted into the mouth of the bulb. The intensity of the explosion varies with the quantity of air present in the bulb."

[1] *Amer. Jour. Med. Sci.*, Sept., 1895.

[2] Koch: *Berlin. klin. Wochenschr.*, No. 15, 1882; *Mitth. a. d. Kais. Gesundheitsamte*, Bd. 2, 1884.

eter. They may become confluent, to form a dense dry, granular mass with irregular surface and of a creamy-white color. The growth is very friable, but coherent, and may be picked up in clumps on the platinum wire. The first generation from tissues is very slow in developing, but succeeding generations grow more rapidly, and may form a wrinkled, dry, cream-colored membranous layer on the surface of the medium.

Morphology.—Slender rods, usually shorter than when observed in sputum, and in fresh cultures staining homogeneously; in older cultures presenting a segmented or irregularly stained appearance. They frequently occur in pairs of short rods and in closely adhering clumps and strands. When once stained with fuchsin or gentian-violet they are not decolorized by treatment with Gabbet's solution or with a 20 per cent. solution of any of the mineral acids, followed by alcohol. In the sputum of pulmonary tuberculosis the bacillus sometimes occurs in filaments which branch. On this account the organism is considered by many to belong to the group of the streptothrices (see p. 216).

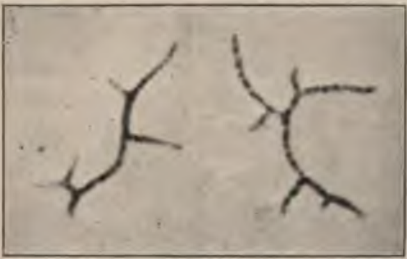

FIG. 57.—Branched tubercle bacilli from sputum; × 2000 (Wright and Brown).

Stained by Gram's method. Not motile. Does not form spores.

Glycerin Agar-agar Slant.—Growth similar to that on blood-serum, but not so vigorous. By continued inoculation of this medium through a number of generations, however, the organism may eventually grow luxuriantly upon it.

Glycerin Bouillon.—Growth on the surface in the form of floating patches or as a membrane similar in appearance to the growth on blood-serum. The growth sinks to the bottom from time to time. The glycerin-bouillon culture is best contained in Ehrlenmeyer flasks, filled to such a depth as to give a wide surface to the fluid and thus permit the access of plenty of oxygen to the growth.

Potato.—The growth is not remarkable.

Agar-agar or bouillon not containing glycerin is not suitable for the cultivation of this bacillus.

Pathogenesis.—The inoculation of guinea-pigs or rabbits by any method is followed by the development of general miliary tuberculosis. Guinea-pigs are most susceptible. These animals usually survive about two or three months, with marked emaciation. The lesions in the spleen and liver in the guinea-pig are characterized by extensive areas of necrosis not confined to the tubercular tissue, large parts of these organs being transformed into a firm yellow, opaque, friable material.

Isolation of the Bacillus Tuberculosis from Tubercular Lesions.—The best method of procedure is to inoculate a guinea-pig subcutaneously in the abdominal wall with tubercular material, and after four to six weeks, when the inguinal lymphatic glands have become enlarged, to kill the animal. The object of killing the animal, rather than allowing it to die spontaneously, is to secure fresh tissue and to avoid the chance of an invasion of the lesions by other bacteria.

Cultures on blood-serum, or on glycerin-agar "slant" tubes, are then to be made from the tuberculous lymphatic glands of the inguinal and retroperitoneal regions of the animal. A number of tubes are to be inoculated, say three or four, from each of the two or three glands, a large quantity of material being spread upon the surface of each tube. Great care is to be exercised to avoid contamination with other bacteria in preparing these cultures. The culture-tubes used should contain freshly prepared moist medium, and immediately after inoculation should be sealed air-tight to prevent evaporation. This may conveniently be done by first cutting off the projecting portion of the cotton stopper and inserting a cork into the mouth of the tube in such a way as to push the cotton stopper before it.

In order to prevent the invasion of fungi from the cotton, the neck of the tube should be heated in the Bunsen flame until the cotton begins to brown before inserting the cork, which should also be charred in the Bunsen flame before insertion. The tubes may also be sealed with wax or paraffin or covered with small rubber caps.

Cultures may also be made from other organs containing tubercular lesions, but the lymphatic glands seem to offer the best examples of large tubercular foci, and therefore are preferred for the purpose in view.

The tubercular lesions in human tissues are not ordinarily favorable for the isolation of the bacillus, on account of the frequent presence of other bacteria in them and because of the small number of tubercle bacilli usually present in tissue otherwise suitable.

We have found the firmly coagulated, opaque blood-serum medium above mentioned to be quite adequate for the cultivation of the bacillus from its lesions, both in man and guinea-pigs.

A *special culture-medium* for the cultivation of the bacillus tuberculosis is that recommended by Lubinski. Its composition and mode of preparation are as follows—to make one liter of medium :

Take 1 kilo of potato in small pieces, and, after thoroughly washing, cook in the steam sterilizer for three or four hours with 1500 c.c. of water. Of the mass thus formed take 1000 c.c., and add to it 500 grams of finely chopped beef, and allow it to stand in the cold for twenty-four hours, then boil the mixture for a few minutes and filter. Add to the filtrate 1 to 1.5 per cent. agar-agar, and boil until the agar-agar is thoroughly dissolved. This will require say half an hour's boiling. Next add 1 per cent. pepton and 0.5 per cent. sodium chlorid. When these are thoroughly in solution, and after about five minutes' continued boiling, the mixture is to be neutralized with sodium hydrate solution, boiling meanwhile. When brought to the proper reaction it is to be filtered, and to the filtrate add 4 per cent. glycerin. The medium is then to be run into test-tubes and sterilized as in the case of agar-agar. The loss by evaporation in boiling is to be made up with water.

A bouillon may be also made by leaving out the agar-agar.

Another *special culture-medium* is that recommended by Capaldi. This consists in mixing with fluid agar-agar in tubes, at a temperature of 45° C., three or four large loopsful of the yelk of a fresh egg to each tube. When the portion of the yelk taken is thoroughly mixed with the fluid agar, the tube is placed on its side, and its contents allowed to become solid by cooling so as to form a "slant." In securing the yelk aseptic precautions must be used. The egg must be quite fresh. By suitable manipulations the yelk is to be freed from the "white" and placed in a small, clean dish. Then the yelk-membrane is to be seared with a hot knife and an opening made in it. Through this opening the yelk is obtained with the loop. Before using the culture-tubes so prepared, they should be tested as to their sterility by placing in the incubator for twenty-four hours.

Occurrence.—In tubercular lesions generally and in the sputum of pulmonary phthisis, in the urine in many cases of genito-urinary tuberculosis, and in the feces in intestinal tuberculosis. The tuberculosis of cattle is generally considered to be due to this organism, while the tuberculosis of birds is probably due to a different variety.

Does not multiply outside of the body except in cultures.

May occur on the surface of objects contaminated with the excreta of tuberculous individuals or in the dust of places inhabited by such individuals.

Diagnosis.—For clinical purposes the tubercle bacillus may be identified in cover-glass preparations by means of the special methods of staining given below.

These methods, as well as many others, depend upon the fact that the bacillus tuberculosis, when once thoroughly stained with an aniline dye, does not give up its stain in the presence of acids, as nearly all other bacteria do. The bacillus tuberculosis may therefore be identified even among a mixture of other bacteria by this property, taken in connection with its morphology, in most of the routine work of the pathological laboratory. Practically, the only other bacilli with which it may be confounded are the bacillus of leprosy and the smegma bacillus, both of which, when stained, resist the decolorizing action of acid. It may be differentiated from the smegma bacillus by the fact that it is not decolorized by alcohol (95 per cent.) after the usual treatment with acid, while the smegma bacillus is decolorized under these circumstances.

As a rule, the differential test with alcohol need only be applied in the examination of urine and the material derived from about the external genitalia, especially in the case of females.

The differentiation from the bacillus of leprosy by certain quantitative differences in staining reactions has been attempted, but it is very unsatisfactory, and it is doubtful if there is as yet any reliable method of distinguishing between these two organisms, considered by themselves.

Examination of Sputum for Tubercle Bacilli.[1]—The morning sputum should be taken for examination. Select one of the dense, grayish-white particles, and with the aid of small pointed forceps or the platinum wire rub it over the surface of a cover-glass, breaking it up as much as possible. The material should be spread in a very thin layer. The preparation is next to be "fixed" in the ordinary way described for cover-glass preparations (see p. 92), and is then to be treated as follows:

1. Stain in carbol-fuchsin solution, steaming for one

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[1] According to Jochmann,* the tubercle bacilli in sputum and urine may be made to multiply, and thus be more easily found, by mixing the sputum or urine with a culture-fluid of the following composition, and placing the mixture in the incubator for twenty-four hours:

"Nahrstoff Heyden,"	5;
Sodium chlorid,	5;
Glycerin,	30;
Water,	1000;
Lactic acid (1 per cent. solution),	10.

This fluid must be sterilized as in the case of other culture-media. 10 c.c. of sputum are mixed with 20 c.c. of this fluid, and after the mixture has stood for twenty-four hours in the incubator 3 c.c. of pure carbolic acid are added. The mixture is then shaken and the sediment examined.

In the case of urine, the sediment obtained by the centrifuge is mixed with sufficient of this culture-fluid to fill the centrifuge-tube, which is then placed in the incubator for twenty-four hours. The sediment is then to be examined.

The virtues of this culture-fluid are said to lie in its property of preventing the rapid multiplication of bacteria other than the tubercle bacillus. The tubercle bacillus, however, does not grow on it as well as on glycerin-agar or blood-serum.

* *Hygienische Rundschau*, No. 20, 1900, and No. 1, 1901.

minute over the Bunsen flame, with the staining solution thoroughly covering all the surface of the cover-glass. None of the surface of the cover-glass should be allowed to become dry by evaporation, as this causes a precipitate to form, but more of the staining fluid should be added from time to time to keep it completely covered as evaporation occurs. The object of the heating is thoroughly to impregnate the bacilli with the dye.

2. Wash in water.

3. Cover with Gabbet's solution for twenty seconds. The solution should also be applied to the uncharged slide of the cover-glass to remove any dried stain which may have collected thereon.

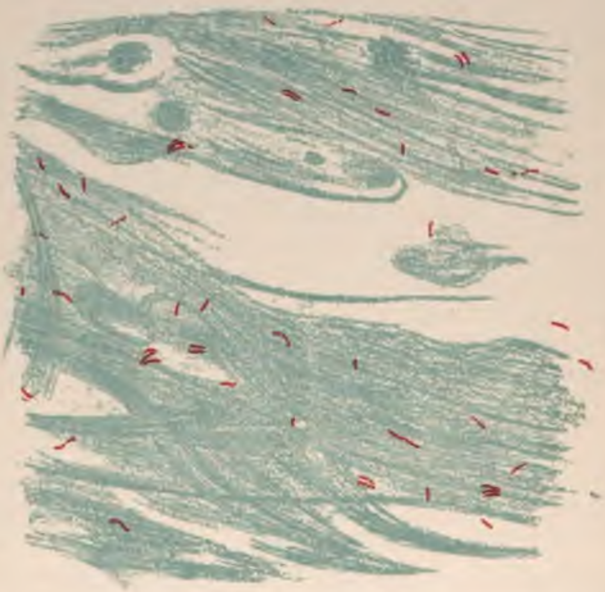

FIG. 58.—Tubercle bacilli in sputum (carbol-fuchsin and methylene-blue).

4. Wash thoroughly in water.

5. Mount in water or balsam. Water is to be preferred, for the reason that the apparent size of the bacilli is larger when examined in water.

Another method is as follows:

1. Stain in carbol-fuchsin solution or aniline-fuchsin solution in the manner above indicated.
2. Wash in water.
3. Decolorize in a solution composed of—

Hydrochloric acid,	3 parts;
Alcohol (70 per cent.),	97 "

until the preparation has a faint pink color.

4. Wash thoroughly in water.
5. Stain for thirty seconds with Löffler's methylene-blue solution without heating.
6. Wash in water and mount.

By both of these methods the tubercle bacilli are stained red, while other bacteria and the nuclei of cells are stained blue (Fig. 58). There are other methods for staining tubercle bacilli, but these fulfil all the requirements of practical work.

Tubercle bacilli, *when present in sputum in very small numbers*, may sometimes be demonstrated by methods of sedimentation. A good means of sedimentation consists in heating the sputum in a test-tube either with boiling water or in the steam sterilizer for fifteen minutes. The heat coagulates the cells and albuminous constituents, which sink to the bottom, carrying with them the bacilli. The supernatant liquid may then be poured off and the sediment examined as above.

Another method of treating the sputum when only a small number of bacilli may be present is as follows: Place 10–15 c.c. of the sputum in a wide-mouthed bottle of 100 c.c. capacity. Add 10 c.c. of water and 6 c.c. of liquefied carbolic-acid crystals. Close the flask with a rubber cork and shake for one minute. After shaking, fill the bottle with water and shake again. Then pour the contents of the bottle into a sedimenting glass, let stand for twelve to twenty-four hours, and examine the sediment. The cover-glass preparations of the sediment before staining are to be washed in ether or chloroform, and then in alcohol, or they may be washed in a mixture of alcohol and ether, equal parts.

In a very few cases of gangrene of the lung bacilli like smegma bacilli have been found in the sputum. These may be mistaken for tubercle bacilli (*vide ante*).

Tubercle Bacilli in Urine.[1]—The sediment of the urine should be examined. This may be rapidly thrown down by the centrifuge.

In urine, smegma bacilli may be mistaken for tubercle bacilli, especially in the urine of females. The smegma bacillus resembles the tubercle bacillus very closely in form, and, like the latter, it retains its stain in the presence of acids. It differs from the tubercle bacillus, however, in that it is decolorized by alcohol. Therefore in staining for tubercle bacilli in the urine alcohol should be used in decolorizing. The procedure is as follows:

1. Make a cover-glass preparation from the sediment.
2. Stain with carbol-fuchsin solution for one minute, as described in the case of sputum.
3. Wash in water.
4. Decolorize with either of the decolorizing solutions given above for sputum, proceeding in the manner there described.
5. Wash thoroughly in water.
6. Wash in alcohol (95 per cent.) thirty seconds.
7. Wash in water.
8. Stain with Löffler's methylene-blue solution twenty seconds, without heat.
9. Wash in water and mount.

The presence of tubercle bacillus in urine may also be shown by the results of the inoculation of a guinea-pig with the sediment.

Surgical Tuberculosis.—The demonstration of the tuberculous nature of material removed at operations may be made by the histological examination, by the demonstration of the tubercle bacilli on cover-glass preparations, as in sputum, and by the results of the inoculation of guinea-pigs with the material. Cultures are not ordinarily practicable.

The *examination of cover-glass preparations* is commonly of little value, owing to the small number of bacilli usually present.

The *histological examination* may be often made very satisfactorily by frozen sections of the tissues. If possible, however, regularly hardened and imbedded tissues, cut in fairly thin sections and stained with hematoxylin and eosin, are preferable.

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[1] See foot-note, p. 175.

The *inoculation of guinea-pigs* is to be made subcutaneously in the abdominal wall, either with a hypodermic syringe if the material is fluid, or, if it be in the form of tissue, by inserting a small piece beneath the skin. Material obtained on a swab may also be used for inoculation by introducing the infected swab beneath the skin and moving it back and forth a few times. If tubercle bacilli are present in the material, the animal will show enlargement of the inguinal lymphatic glands in about three weeks and will usually die of miliary tuberculosis in the course of six to ten weeks. If necessary, the glands in the inguinal region may be examined histologically after three weeks for the presence of tubercular lesions, or examined by cover-glass preparations for tu-

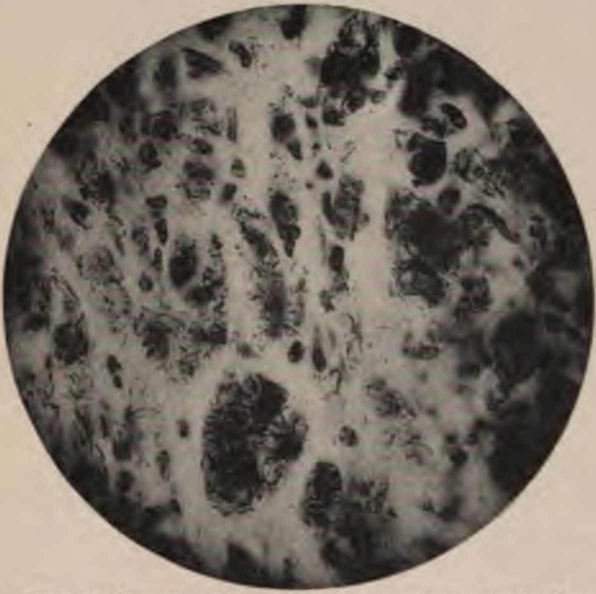

FIG. 59.—Bacillus of leprosy: section through a subcutaneous node, showing the bacilli in tissue-cells; × 500 (Fränkel and Pfeiffer).

bercle bacilli. The discharges from sinuses, etc. may also be tested for the presence of tubercle bacilli as above indicated, the material being obtained on a "swab."

Leprosy.—The bacillus of this disease shows essentially the same staining reactions as the bacillus tuberculosis. In sections of the lesions the bacilli are found in large numbers, mostly inside the tissue-cells (see Fig. 59).

Spirillum of Asiatic Cholera (Comma Bacillus).[1]—*Morphology* (Figs. 60, 61).—In fresh cultures the organism appears usually as a slightly curved rod somewhat shorter than the tubercle bacillus, but much thicker. The curving of the rod varies, being very marked in some individuals and absent in others. Sometimes two rods are joined end to end with their convexity pointing in opposite directions, or moderately long, undulating threads may be found. It seems probable that the curved rods represent the segments of a spirillum, and hence the name of the organism.

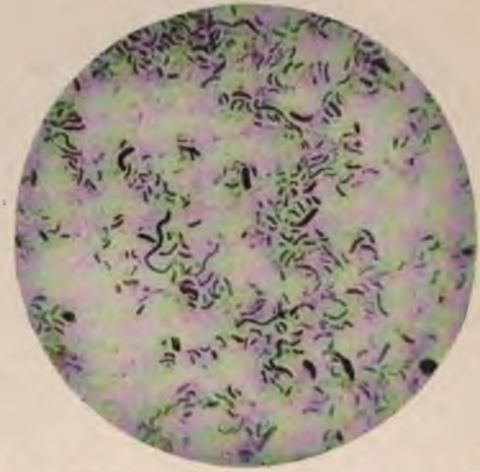

FIG. 60.—Spirillum of Asiatic cholera, from a bouillon culture three weeks old, showing long and degenerate forms; × 1000 (Fränkel and Pfeiffer).

In cultures some days old degenerated and atypical forms are found (involution forms). The organism is motile, and a single flagellum is attached to the end of the rod.

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[1] Koch; *Deutsche med. Wochenschr.*, 1884 and 1885.

It is not stained by Gram's method.

Colonies on Gelatin Plates (Fig. 62).—After twenty-four to forty-eight hours at a temperature of 20° to 22° C. the largest colonies will appear as masses of indefinite granular material lying in circular areas of liquefied gelatin in which granular shreds are scattered. Within the next twenty-four hours the areas of liquefaction increase, and the colonies appear under the low power "as a dense granular mass surrounded by an area of liquefaction through which can be seen granular prolongations of the colony, usually extending ir-

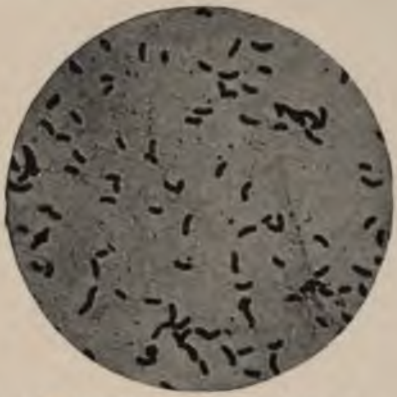

FIG. 61.—Spirillum of Asiatic cholera, showing the flagella; × 1000 (Günther).

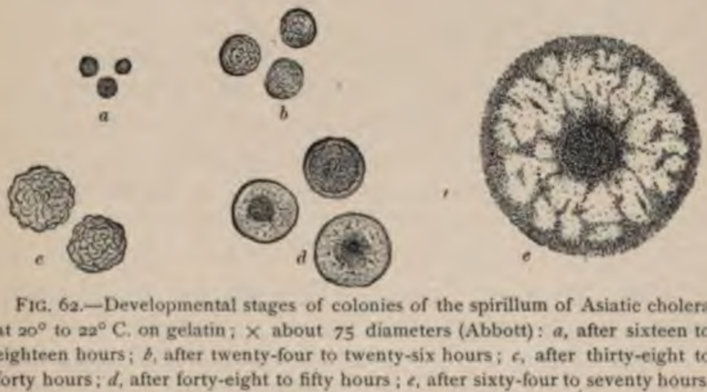

FIG. 62.—Developmental stages of colonies of the spirillum of Asiatic cholera at 20° to 22° C. on gelatin; × about 75 diameters (Abbott): *a*, after sixteen to eighteen hours; *b*, after twenty-four to twenty-six hours; *c*, after thirty-eight to forty hours; *d*, after forty-eight to fifty hours; *e*, after sixty-four to seventy hours.

regularly between the periphery and the central mass" (Abbott), while the margin of the liquefied area is marked by delicate radiating filaments closely packed together.

The colonies on agar-agar plates are not characteristic. Growth is rapid.

Gelatin Stab.—Growth all along the line of inoculation with liquefaction at the surface in funnel form after forty-eight hours. The liquefaction proceeds in such a manner that the liquefied area has a smaller diameter at the surface than immediately beneath, and, owing to the fact that the liquefied gelatin does not fill the cavity, a space is left between the surface of the medium and the surface of the liquefied gelatin so that the appearance of an air-bubble is produced. Along the deeper portions of the line of inoculation the liquefaction is slow.

Bouillon.—Diffusely clouded. A thin pellicle forms on the surface after a time.

Litmus-milk.—Turned red and coagulated.

Indol-production.—In cultures in Dunham's pepton solution or in the pepton solution of Koch (2 per cent. pepton and 1 per cent. sodium chlorid) a rose-color is produced by the addition of sulphuric acid alone. (Concentrated c. p. acid is to be employed, as in the test for indol-production by the bacillus coli communis.) The production of the rose-color without the addition of the sodium nitrite shows that nitrites as well as indol are formed by the growth of the organism in the pepton solution. The reaction can be obtained in cultures which have been but eight hours in the incubator.

Potato.—Thin, dry, grayish-white growth which does not spread over the surface.

Pathogenesis.—The pathogenic effects of the cholera spirillum are best shown by the inoculation of guinea-pigs. There are two methods of inoculation, as follows:

1. *The Method of Pfeiffer.*—Scrape from the surface of a fresh agar-agar culture as much of the growth as will adhere to a platinum wire bent into the form of a small loop. Suspend this amount of material in 1 c.c. of bouillon, and inject the suspension into the peritoneal cavity of a guinea-pig

by means of a hypodermic syringe. With virulent cultures this inoculation soon produces a fall in the temperature of the animal, which continues and becomes more marked, death occurring in from twelve to twenty-four hours. At the autopsy of the animal a clear fluid will be found in the peritoneal cavity and in the thorax.

2. *The Method of Koch.*—This depends upon the fact that the animal may be infected through the alimentary canal, provided the acidity of the gastric juice be neutralized, this acidity being destructive to the cholera spirillum.

A soft catheter is passed into the stomach of the animal through the mouth, and through this 5 c.c. of a 5 per cent. solution of sodium carbonate is injected. After ten or fifteen minutes 10 c.c. of a bouillon culture of the organism are injected through the catheter, and immediately afterward the animal receives subcutaneously 1 c.c. of the tincture of opium for every 200 grams of its body-weight. The object of this opium administration is to stop peristalsis, so that the organisms may be longer in contact with a given area of the mucous membrane of the intestine. The result of the inoculation first appears after about twenty-four hours. The animal then has no appetite and is listless. Later, paralysis of the hinder extremities appears, respiration is prolonged and weak, the heart-beats become feeble, and the body-temperature may become subnormal. Death usually occurs after the animal has been a few hours in this condition.

At the autopsy the small intestine will be found to be injected and containing a flocculent colorless fluid in which comma bacilli are present in great numbers.

Serum Reaction.—Cholera spirilla cease their motion, and aggregate together in clumps and masses when a bouillon culture of them is mixed with the serum of an animal immunized toward them. This phenomenon is called the serum reaction. Moreover, if cholera spirilla be placed in the peritoneal cavity of an immunized guinea-pig, they will rapidly undergo disintegration, and will be destroyed. This is called Pfeiffer's phenomenon.

Occurrence.—In the alvine dejections and in the intestinal contents of cholera patients (Fig. 63). It apparently only rarely invades the circulating blood. Its presence in the vomitus may sometimes be shown. It has been found in the water-supplies during epidemics.

The cholera spirillum is the representative of a large group of spirilla, many of which may be found in river waters. According to Abbott and Bergey, the only trustworthy method of distinguishing some of these from the true cholera spirillum is their failure to manifest a "clump reaction" with the serum of an animal immunized to infection with the true cholera spirillum.

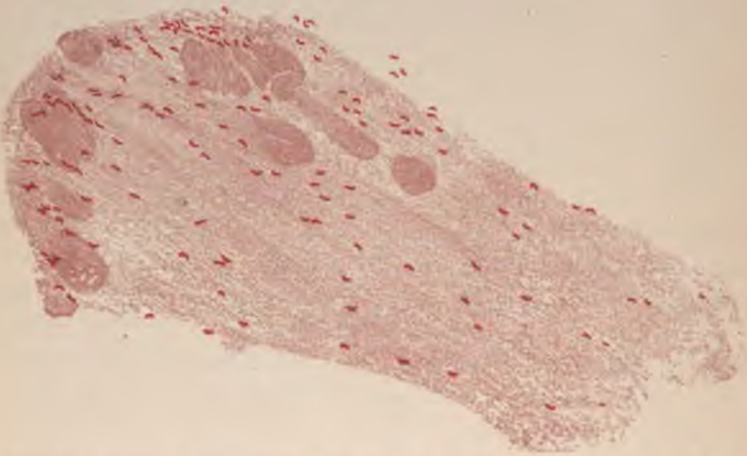

FIG. 63.—Cover-glass preparation of a mucous floccule in Asiatic cholera; × 650 (Vierordt).

Bacteriological Diagnosis.[1]—Because of the manifold channels which are open for the dissemination of this disease it is of the utmost importance that its true nature should be recognized as quickly as possible, for with every moment of delay in its recognition opportunities for its spread are multiplying. It is essential, therefore, when employing bacteriological means in making the diagnosis to bear in mind those biological and morphological features of the organism that appear most quickly under artificial methods of cultivation, and which at the same time may be considered as characteristic of it—viz. its peculiar morphology and grouping; the much greater rapidity of its growth over that of other bacteria with which it may be associated; the characteristic appearance of its colonies on gelatin plates and of its growth in stab-cultures in gelatin; its property of producing indol and coincidently nitrites in from six to eight hours in pepton solution at 37° to 38° C.; and its power of causing the death of guinea-pigs in from sixteen to twenty-four hours when introduced into the peritoneal cavity, death being preceded by symptoms of extreme toxemia, characterized by prostration and gradual and continuous falling in temperature of the animal's body.

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[1] Abbott: *Principles of Bacteriology*.

In a publication recently made by Koch[1] he called attention to a plan of procedure that is employed in this work in the Institute for Infectious Diseases in Berlin. In this scheme the points that have been enumerated are taken into account, and by its employment the diagnosis can be established in the majority of cases of Asiatic cholera in from eighteen to twenty-two hours. In general, the steps to be taken and the points to be borne in mind are as follows. The material should be examined as early as possible after it has been passed:

1. Microscopic examination. From one of the small slimy particles that will be seen in the semifluid evacuation prepare a cover-slip preparation in the ordinary way and stain it. If, upon microscopic examination, only curved rods or curved rods greatly in excess of all other forms are present, the diagnosis of Asiatic cholera is more than likely correct; and particularly is this true if these organisms are arranged in irregular linear groups, with the long axis of all the rods pointing in nearly the same direction; that is to say, somewhat as minnows arrange themselves when swimming in schools up stream (Koch).

In 1886, Weisser and Frank[2] expressed their opinion upon the value of microscopic examination in these cases in the following terms:

(*a*) In the majority of cases microscopic examination is sufficient for the detection of the presence of the comma bacillus in the intestinal evacuations of cholera patients.

(*b*) Even in the most acute cases, running a very rapid course, the comma bacillus can always be found in the evacuations.

(*c*) In general the number of cholera spirilla present is greater the earlier death occurs; when death is postponed and the disease continues for a longer period, their number is diminished.

(*d*) Should the patient not die of cholera, but from some other disease, such as typhoid fever, that may be engrafted upon it, the comma bacilli may disappear entirely from the intestines.

2. With another slimy flake prepare a set of gelatin plates. Place them at a temperature of from 20° to 22° C., and at sixteen, twenty-two, and thirty-six hours observe the appearance of the colonies. Usually at about twenty-two hours the colonies of this organism can easily be identified by one familiar with them.

3. With another slimy flake start a culture in a tube of pepton

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[1] *Zeitschrift f. Hygiene u. Infectionskrankheiten*, Bd. 14, 1893.

[2] *Ibid.*, Bd. 1, p. 397.

solution—either the solution of Dunham or, as Koch proposes, a solution of double strength of that of Dunham (Witte's pepton is to be used, as it gives the best and most constant results). Place this at 37° to 38° C., and at the end of from six to eight hours prepare cover-slips from the *upper layers* (without shaking) and examine them microscopically. If comma bacilli are present and capable of multiplication, they will be found in this locality in almost pure culture. After doing this prepare a second pepton culture from the *upper layer*, also a set of gelatin plates, and with what remains make the test for indol by the addition of 10 drops of concentrated sulphuric acid for each 10 c.cm. of fluid contained in the tube. If comma bacilli are growing in the tube. the rose color characteristic of the presence of indol should appear.

By following this plan "a bacteriologist who is familiar with the morphological and biological peculiarities of this organism should make a more than *probable* diagnosis at once by microscopic examination alone, and a *positive* diagnosis in from twenty to, at the most, twenty-four hours after beginning the examination" (Koch).

There are certain doubtful cases in which the organisms are present in the intestinal canal in very small numbers, and microscopic examination is not, therefore, of so much assistance. In these cases plates of agar-agar, of gelatin, and cultures in the pepton solution should be made.

The plates of agar-agar should not be prepared in the usual way, but the agar-agar should be poured into Petri dishes and allowed to solidify, after which one of the slimy particles may be smeared over its surface. The comma bacillus being markedly aërobic, develops very much more readily when its colonies are located upon the surface than when they are in the depths of the medium. A point to which Koch calls attention in connection with this step in the manipulation is the necessity for having the surface of the agar-agar free from the water that is squeezed from it when it solidifies, as the presence of the water interferes with the development of the colonies as isolated points and causes them to become confluent. To obviate this, he recommends that the agar-agar be poured into the plates and the water allowed to separate from the surface at the temperature of the incubator before they are used. It is wise, therefore, when one is liable to be called on for such work as this to keep a number of sterilized plates of agar-agar in the incubator ready for use, just as sterilized tubes of media are always ready and at hand. The advantage of using the agar plates is the higher temperature at which they can be kept, and consequently a more favorable condition for the development of the colonies. As soon as isolated colonies appear they should be examined microscopically for the presence of organisms having the morphology of the one for which we are seeking, and as soon as such is detected gelatin plates and cultures in pepton solution (for the indol reaction) should be made. The pepton cultures started from the original material should be examined microscopically from hour to hour after the sixth hour that they have been in the incubator. The material taken for examination should always come from *near the surface* of the fluid, and care should be taken not to shake the tube. As soon as comma bacilli are detected in anything like considerable numbers in the upper layers of the fluid, agar-agar plates and fresh pepton cultures should be made from them. The colonies will develop on the agar-agar plates at 37° C. in from ten to twelve hours to a size sufficient for recognition by microscopic examination, and from this examination an opinion can usually be given. This opinion should always be controlled by cultures in the pepton solution made from each of several single colonies, and finally the test for the presence or absence of indol in these cultures.

In all doubtful cases in which only a few curved bacilli are present or in which irregularities in either the rate or mode of their development occurs, pure cultures should be obtained by the agar-plate method and the method of cultivation in pepton solution as soon as possible, and their virulence tested upon animals. For this purpose cultures upon agar-agar from single colonies must be made. From the surface of one of such cultures a good-sized wire-loopful should be scraped, and this broken up in about 1 c.c. of bouillon, and the suspension thus made injected by means of a hypodermic syringe directly into the peritoneal cavity of a guinea-pig of about 350 to 400 grams weight. For larger animals more material should be used. If the material injected is from a *fresh* culture of the cholera organism, toxic symptoms at once begin to appear; these have their most pronounced expression in the lowering of temperature, and if one follows this decline in temperature from time to time with the thermometer, it will be seen to be gradual and continuous from the time of injection to the death of the animal, which occurs in from eighteen to twenty-four hours after the operation.[1]

In general, this is the procedure employed at Berlin in the Institute for Infectious Disease under Koch's direction.

Bacillus of Anthrax.[2] — *Blood-serum.* — Irregularly rounded colonies, several mm. in diameter after twenty-four hours in the incubator. The colonies are grayish, finely granular, and have the appearance of being made up of a dense network of delicate fibrillæ. The blood-serum is slowly liquefied.

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[1] Pfeiffer: *loc. cit.*

[2] Pasteur: *Bull. Acad. de. Méd.*, Paris, T. viii., 1879; Koch: *F. Cohn's Beitr. z. Biol. d. Pfl.*, Bd. 2, 1876.

Morphology.—The organism grows in long segmented threads, the segments varying in length, but usually being two or three times as long as broad and having square or slightly concave ends. These segments represent the bacillus, which is among the largest of the bacteria (Fig. 64).

Pathogenesis.—Mice, guinea-pigs, and rabbits inoculated subcutaneously die with a general invasion of the blood by the organism. Mice are most susceptible to the infection, dying in about twenty-four hours, while guinea-pigs and rabbits survive longer.

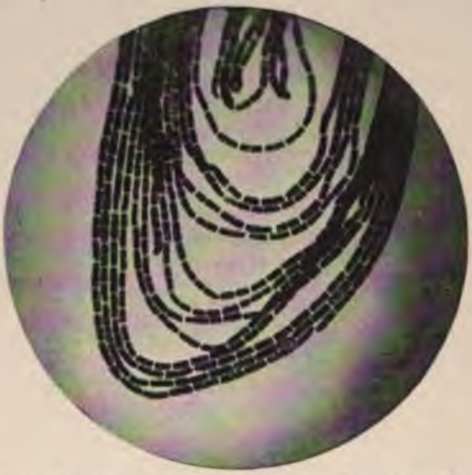

FIG. 64.—Bacillus of anthrax: portion of a colony three days old upon a gelatin plate; × 1000 (Fränkel and Pfeiffer).

In all these animals the most striking lesion is a large soft spleen, and in the guinea-pig also an extensive inflammatory edema of the subcutaneous tissues. On microscopic examination the bacilli will be found in the organs and blood of the heart. If the animal has been dead some time, the number of bacilli present in these situations will be very great, owing to the post-mortem growth. It is characteristic of the bacillus of anthrax in cover-slip preparations from infected tissues that it should have a narrow capsule (Fig. 65) and show square or slightly concave ends. The capsule is not present in cultures.

Stained by Gram's method. Not motile.

Forms oval spores in the middle of the short segments or rods. The spores may be seen in blood-serum cultures after forty-eight hours in the incubator (Fig. 66).

Gelatin Stab.—Growth along the line of stab, with radiating filaments extending laterally into the gelatin, which is slowly liquefied in funnel form (Fig. 67).

Bouillon.—Growth in the form of cotton-like flakes and filamentous masses. No clouding of the medium.

Agar-agar.—Matted network of translucent filaments. Under a lower magnifying power the growth is seen to be made up of twisted and contorted masses of filaments, giving the appearance of curled hair (Fig. 68).

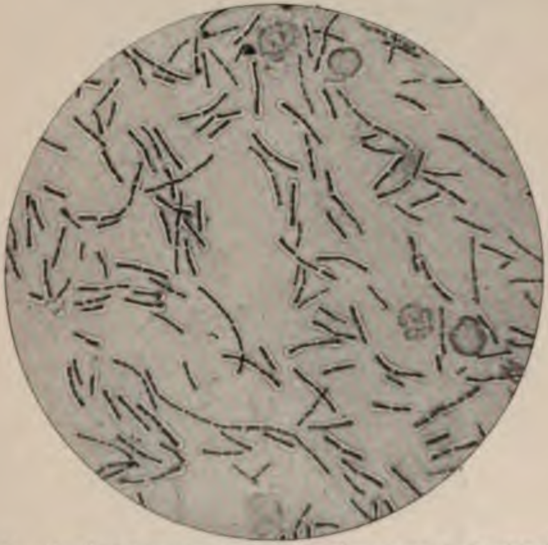

FIG. 65.—Bacillus of anthrax from spleen of a mouse (L. Frothingham).

Potato.—Grayish-white, rather thick, dry layer, having the appearance of frosted glass.

Occurrence.—In malignant pustules, wool-sorter's disease, and intestinal anthrax. Found in the blood of animals dead of anthrax. In man the infection is usually localized at first at the point of inoculation, either on the skin or on the mucous membrane of the air-passages or intestinal tract. Later, a general invasion of the blood may occur and a fatal septicemia result. The organism or its spores may be present in wool or hides, and infection may take place from these.

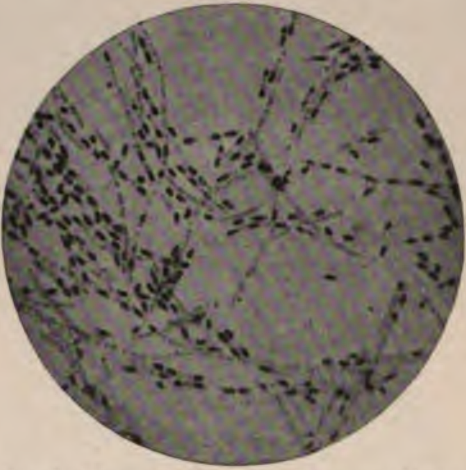

FIG. 66.—Bacillus of anthrax, stained to show the spores; × 1000 (Fränkel and Pfeiffer).

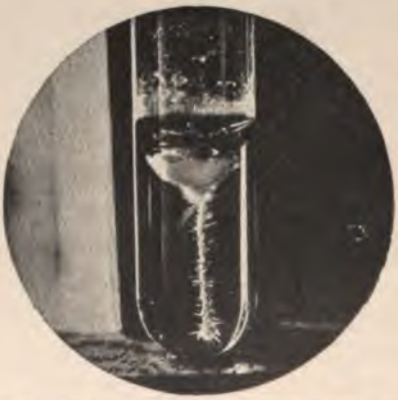

FIG. 67.—Bacillus of anthrax: gelatin stab-culture seven days old (Günther).

Diagnosis.—The bacilli may be found by the cover-glass examination of the contents of the small blebs and vesicles. The bacillus of anthrax may be identified by its morphology (see p. 188), its special characteristics being its large size and its square or concave extremities.

The inoculation of a mouse at the root of the tail with some of the material from the pustule, and the production of the characteristic fatal septicemia, will render the identification certain.

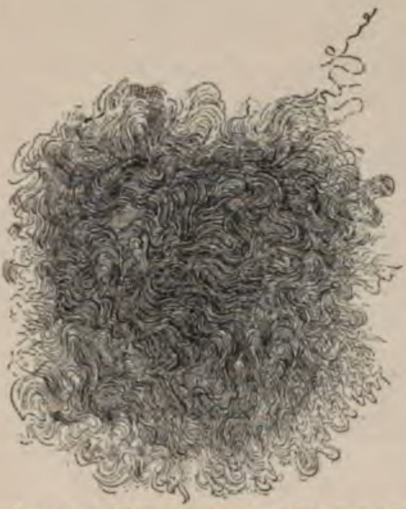

FIG. 68.—Colony of bacillus of anthrax, slightly magnified (Flügge).

Blue

Bacillus Pyocyaneus (Bacillus of Green Pus).[1]—Colonies on *blood-serum* grow rapidly, are not especially characteristic in form, and liquefy the medium, imparting to it a dark greenish color.

Morphology.—Small bacilli with rounded ends (Fig. 69). Decolorized by Gram's method (Welch). Motile, and is provided with a flagellum at one end. Does not form spores.

Gelatin Stab.—Liquefaction in funnel form, with green fluorescence of the upper portions of the medium. The liquefied gelatin is densely clouded, and there may be a viscid pellicle on the surface.

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[1] Gessard: *Annales de l'Institut Pasteur*, T. 5, 1891.

Agar-agar Stab.—A green fluorescence in the upper layers of the medium, which later becomes a dark blue-green.

Potato.—Slightly elevated, brownish, viscid layer. The potato in some cases assumes a green color, in others a brown color. In some cultures the potato when touched with the platinum wire takes on a green color at the point touched. This is the so-called "chameleon phenomenon," and it is best observed in cultures several days old.

Bouillon.—The growth is in the form of flocculi and a delicate surface pellicle. The fluid acquires a green color.

Litmus-milk.—Acid reaction with coagulation.

Dunham's Pepton Solution.—Indol is produced.

Colonies on Gelatin Plates (Fig. 70).—Development is rapid. "Young colonies are provided with a fringe of delicate filaments about their periphery. . . . As growth progresses and liquefaction becomes more advanced, the central mass of the colony sinks into the liquefied depression, while at the same time there is an extension of the colony laterally. . . . At this stage the colony, when slightly magnified, may present various appearances, the most common being that shown in Fig. 70. The gelatin between the growing colonies takes on a bright yellowish-green color, but, as growth is comparatively rapid, it is quickly entirely liquefied, and one often sees the colonies floating about in the pale-green fluid."[1]

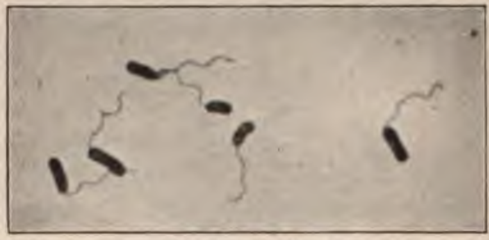

FIG. 69.—Bacillus pyocyaneus showing flagella, from a preparation stained by Dr. Hugh Williams; × 2000 (Wright and Brown).

Pathogenesis.—Subcutaneous inoculation of guinea-pigs and rabbits with 1 c.c. of a virulent bouillon culture may produce purulent infiltration and inflammatory edema of the tissue about the point of inoculation, and death may follow in eighteen to thirty-six hours. Intraperitoneal inoculation may result in a sero-fibrinous or purulent peritonitis with fatal result. In fatal inoculations the bacillus is found in the various viscera, but not in great numbers. Animals inoculated with small amounts may survive with merely local lesions, and an immunity may be produced.

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[1] Abbott: *Principles of Bacteriology.*

Several varieties of this bacillus have been described, but their differences do not seem to be of sufficient importance to justify their separation into distinct species.

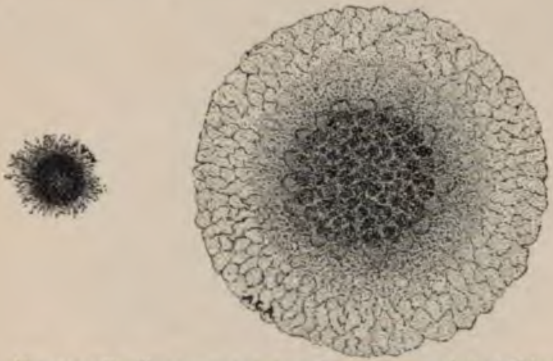

FIG. 70.—Bacillus pyocyaneus: colonies upon gelatin (Abbott).

Occurrence.—" Is widely distributed, occurring often on the human skin, in the feces, and outside of the body. In wounds, stains the dressings bluish-green and produces a somewhat characteristic offensive odor.

" Increases suppuration of wounds, usually with little constitutional disturbance. Is found not infrequently in perforative peritonitis and appendicitis, sometimes in phlegmons, otitis media, broncho-pneumonia, and inflammation of serous membranes, associated usually with other bacteria.

" It was found by H. C. Ernst in tuberculous pericarditis. Often found in diarrheal and dysenteric discharges. May cause general infection in human beings. With or without general infection it may cause hemorrhagic and necrotic enteritis, a form of pyocyaneous infection in human beings which we have repeatedly observed at autopsy. Instances of invasion of the body from wounds by the bacillus pyocyaneus have not been observed" (Welch).

Bacillus of Bubonic Plague.—*Morphology.*—In the tissues the organism occurs as a medium-sized short bacillus with rounded ends. In cultures its size and length vary and its median portion may be swollen so that an ovoid form is produced; it may grow in pairs and in chains, and it may occur as long, thread-like forms. Involution forms of elliptical or round shape, and often of large size, sometimes resembling yeast-cells, are frequent in old cultures or in cultures on special media. These involution forms are easily produced by cultivation on agar-agar containing 2½ to 3½ per cent. of sodium chlorid.

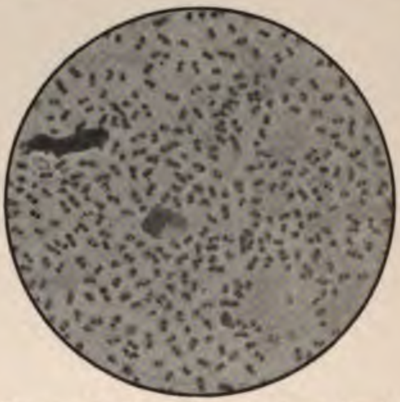

FIG. 71.—Bacillus of bubonic plague (Yersin).

Staining.—The organism stains with the usual aniline dyes, and is decolorized by Gram's method of staining. In the tissues it stains more deeply at its extremities than at its central portions, and it sometimes appears to possess a capsule. The polar staining may sometimes be brought out in cultures by weak staining solutions or by decolorization by alcohol. It is not motile, and it does not form spores.

Gelatin Plates.—The colonies on the surface appear after twenty-four to forty-eight hours at 22° C. They are flat, round, and white or yellowish white in color. Under a low magnifying power the central portion of the colony is granular, while the marginal portion is clear. The colonies do not spread over the surface of the medium.

Gelatin Stab.—Growth all along the line of inoculation with the formation of a layer of growth at the surface of a whitish color. There is no liquefaction of the gelatin.

Gelatin Slants.—A whitish or slightly yellowish layer presenting nothing characteristic.

Agar-agar Plates.—The colonies on the surface appear first as dew-drops, and have already attained their maximum development after twenty-four to forty-eight hours in the incubator. They will then grow white in color, and present an opalescent or iridescent margin. Under the microscope they are distinctly granular. Considerable difference in size may be observed among the colonies. The larger colonies are said to be less virulent for animals than the smaller colonies, and it is claimed that these larger colonies when transplanted give rise to large colonies again.

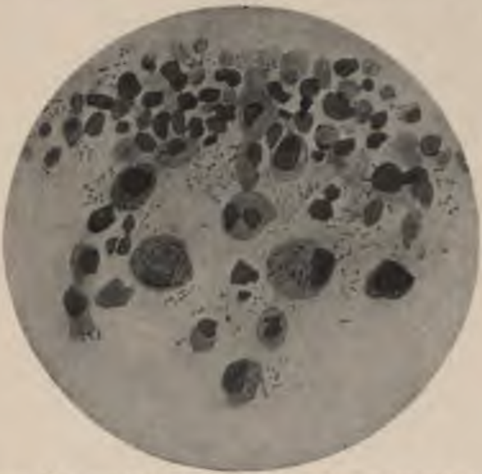

FIG. 72.—Bacilli of plague and phagocytes, from human lymphatic gland; × 800 (Aoyama).

Agar-agar Slant.—The colonies tend to become confluent, and the growth is somewhat viscid.

Bouillon.—The fluid usually remains clear, and the growth appears in the form of a granular or flocculent sediment which may here and there adhere to the wall of the tube. In bouillon cultures richly inoculated and retained in a perfectly and undisturbed position at room-temperature for some days a characteristic appearance is produced. In twenty-four to forty-eight hours islands of growth appear underneath the surface in the form of flakes. In the next twenty-four to forty-eight hours there grow down into the fluid from the flakes long, stalactite-like masses, the liquid remaining clear. In four to six days the islands of growth have become more compact and solidified. If the flask be now slightly disturbed, the islands fall to the bottom, bringing with them the stalactite-like growths. The latter are very fragile. In addition to these appearances there is a deposit of growth on the wall of the flask and at the bottom, as well as a ring of growth on the margin of the surface of the liquid.

Milk.—Growth without coagulation.

No production of indol.

In neutral litmus bouillon the blue color is changed to red.

There is no odor, and no pigment production.

The organism is aërobic.

It remains alive in cultures for five to six weeks at least.

Growth occurs at all temperatures from 4° C. to 37° C. The best temperature for growth is 30° to 32° C.

Pathogenesis.—The organism is pathogenic for a great variety of animals, including mice, rats, guinea-pigs, and rabbits. In these animals death generally follows in from two to six days after subcutaneous inoculation. The lesions produced are hemorrhagic edema at the seat of inoculation, enlargement of the lymphatic glands with more or less hemorrhage, enlargement of the spleen and its follicles. The bacilli are present in large numbers in the enlarged lymphatic glands and in the internal organs; they are less numerous in the blood. Rats and certain other animals may be infected by feeding. Pigeons, chickens, and cattle are immune.

Occurrence.—The bacillus is found in large numbers in the buboes, pustules, pulmonary lesions, and other localized lesions of the bubonic plague. It also may be found in larger or smaller numbers in the blood and internal organs generally, and it may be present in the sputum, bile, and alvine discharges. The pus of the buboes which break spontaneously may be sterile. The organism may be demonstrated in the circulating blood of cases of plague.

Bacteriological Diagnosis.—In cases of suspected plague the bacillus is to be sought for in the blood and in the buboes. In cases of pneumonia the sputum especially is to be examined. In the examination cultures as well as cover-glass preparations are to be used.

Perhaps the most certain method of identification of the bacillus is the inoculation of the mucous membrane of the nose of the rat. The simple rubbing of a portion of the culture upon the mucous membrane appears to be sufficient to produce a fatal result in the rat if the culture is that of the genuine bacillus. As a culture-medium agar-agar or blood-serum is to be used in cases where there is no mixed infection. If there is mixed infection of the material to be examined, gelatin surface-cultures are to be made.

The inoculation of animals for diagnostic purposes should be made with the greatest precaution to prevent the spread of the disease.

Bacillus of Influenza.[1]—*Morphology.*—Very small bacilli, with rounded ends and of variable length, sometimes growing into long forms, more or less bent or curved (see Fig. 73).

Stains more deeply at the ends than in the middle, and in the long forms shows irregularity of staining. The faintly stained areas are very sharply defined, as in the case of the typhoid bacillus.

In cover-glass preparations from bronchial secretions (see Fig. 74) the bacillus appears smaller and less plump than it does in preparations from cultures. It also does not show irregularities in staining.

Cultivation.—Does not grow in the ordinary culture-media, but may be cultivated on agar-agar "slants," the surfaces of which have been smeared with a few drops of sterile

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[1] Pfeiffer: *Zeitschrift f. Hygiene u. Infectionskrankheiten*, Bd. 13, 1893.

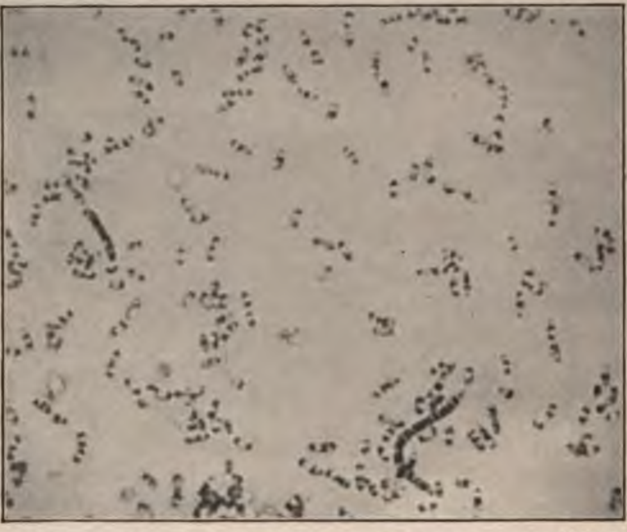

FIG. 73.—Influenza bacilli from a culture on blood-agar; × 2000 (Wright and Brown).

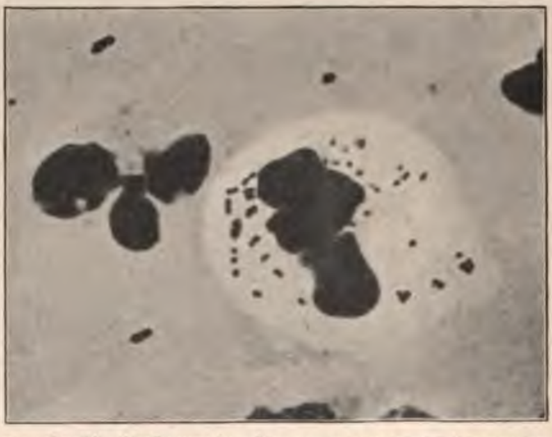

FIG. 74.—Bacilli of influenza in a leucocyte in a cover-glass preparation from sputum. A pneumococcus also in the same leucocyte and other pneumococci free. The small size of the bacillus of influenza will be apparent by comparison with the pneumococci; × 2000 (Wright and Brown).

blood. The blood of man, rabbits, guinea-pigs, pigeons, or frogs will serve for this purpose, the best growth being obtained with pigeon's blood. The blood may be obtained from a needle-prick, and spread over the surface of the agar-agar by means of the platinum loop. The skin should be previously thoroughly washed with alcohol and ether, and the first drops of blood should not be used. Human blood is best obtained from the lobe of the ear or from the finger. Tubes thus prepared are only rarely contaminated.

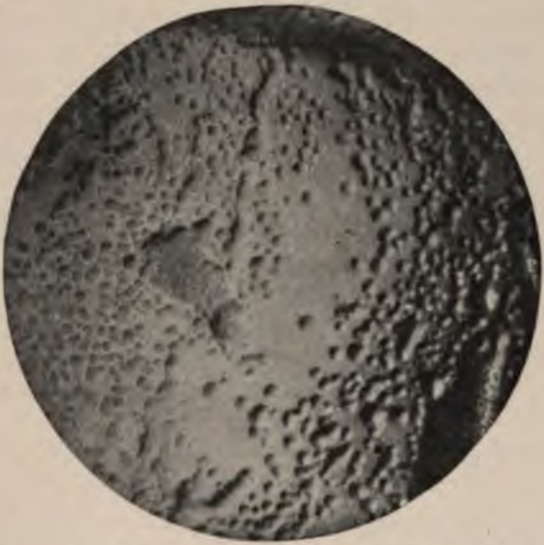

FIG. 75.—Bacillus of influenza: colonies on blood agar-agar; low magnifying power (Pfeiffer).

Colonies.—After twenty-four hours in the incubator the colonies appear as minute colorless, glassy, transparent points resembling small drops of dew. They never attain any size, and do not become confluent. They are barely visible to the unpractised eye, and require a low magnifying power to be seen clearly. Under the low magnifying power they are translucent, homogeneous, not granular, and circular in outline (Fig. 75).

Decolorized by Gram's method. Not motile. Will not grow without oxygen.

Pathogenesis.—The ordinary laboratory animals are not susceptible to infection with this organism.

Occurrence.—Found in the exudate of the respiratory tract in influenza, frequently inside of leucocytes (Fig. 74). It may be present in the small bronchi and in the exudate of broncho-pneumonia in this disease, sometimes unaccompanied by other organisms. It has been observed in purulent meningitis secondary to influenza.

Diagnosis. — Microscopical examination of cover-glass preparations of the bronchial sputum shows very small, short, round-ended bacilli, often in very large numbers and frequently in the pus-cells. These bacilli frequently occur in pairs, and resemble pairs of cocci. Their ends may be more deeply stained than the central portions. For the staining of cover-glass preparations of the sputum Pfeiffer recommends that a very dilute carbol-fuchsin solution be applied for five to ten minutes. The cover-glass preparation is to be made from a distinctly purulent portion of the sputum. Staining with Löffler's methylene-blue solution also gives good results. See also W. H. Smith's method for staining the capsule of the pneumococcus, page 140.

The bacillus of influenza may be cultivated from the sputum by breaking up a small portion of a distinctly purulent character in 1 or 2 c.c. of bouillon, and then spreading a platinum loopful of the suspension over the surface of a blood-agar-agar slant, which is then placed in the incubator. After eighteen to twenty-four hours the characteristic colonies may be visible with the aid of a hand-lens. These should not grow in ordinary media unless blood or hemoglobin be present, and should have the morphology of the bacillus of influenza.

Bacillus of Glanders (Bacillus Mallei).[1]—*Blood-serum.*—Rounded, elevated, colorless, viscid-looking colonies, growing slowly and becoming well developed after thirty-six hours in the incubator. They may attain a diameter of 2 or 3 mm., and after a time they assume a brownish tint.

Morphology.—Bacilli of medium size, variable in length, having round or conical ends, and frequently showing faintly stained areas in their protoplasm (Fig. 76). The larger forms of the bacillus are usually slightly bent or wavy in outline. Slight irregularities in shape may be observed. The morphology varies considerably on different culture-media.

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[1] Löffler: *Arbeiten a. d. Kais. Gesundheitsamte*, Bd. 1, 1886.

In cover-glass preparations from the lesions the bacilli usually appear somewhat longer and thicker than the tubercle bacillus, and show numerous sharply defined, unstained, or faintly stained areas in their protoplasm (Fig. 77). They have rounded or conical ends, and are sometimes slightly irregular in shape. As a rule, they are present in small numbers. If Löffler's methylene-blue solution is used for staining the cover-glass, it should be heated; if carbol-fuchsin is used, it should be followed by a slight decolorization with 95 per cent. alcohol to better differentiate the bacilli.

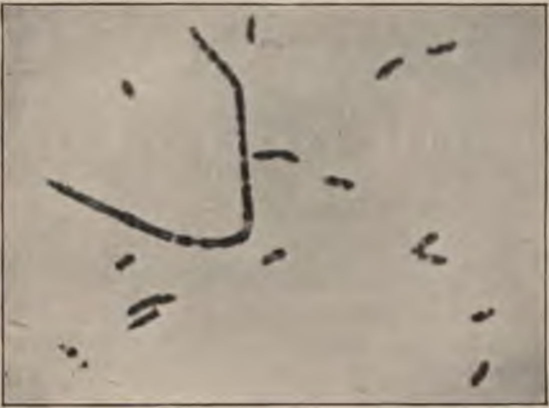

FIG. 76.—Glanders bacilli from a young culture on potato; × 2000 (Wright and Brown).

Potato.—After thirty-six hours in the incubator a rather thick, colorless, viscid-looking layer appears, which soon assumes a brownish tint and resembles honey in appearance. Later the brown color changes to a dark reddish-brown, and the growth becomes thicker and more opaque, while the potato takes on a dark-gray color.

Pathogenesis.—When inoculated subcutaneously into guinea-pigs the characteristic result is swelling and inflammation of the scrotum, appearing after a variable number of days, often about a week.

The animals usually survive several weeks, with ulceration at the point of inoculation. The lesions produced consist in suppurative processes or abscess-formations in or about the testes, in the lymph-glands, in the anterior nares, about the joints, and in other situations, besides small grayish nodules or areas in the viscera—the so-called "glanders tubercles." The suprarenal capsules usually show red areas, and they may be enlarged. On microscopic examination, the small nodules as well as the extensive suppurative areas will be found to be composed of necrotic material containing leucocytes and fragments of chromatin. The distribution and extent of the lesions vary with each animal, but the involvement of the testis or its membranes is practically constant and pathognomonic of the bacillus of glanders. This involvement of the testis may consist, in early cases, in the presence of yellow foci in or about the tunica vaginalis, or in later cases the organ may show large yellow areas with purulent softening.

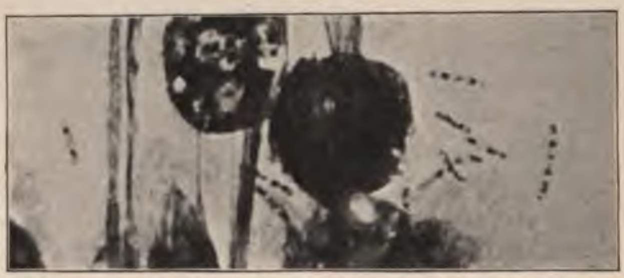

FIG. 77.—Glanders bacilli in a cover-glass preparation from a lesion in a guinea-pig, showing the marked irregularity in the staining of the bacilli; × 2000 (Wright and Brown).

Intraperitoneal inoculation with virulent cultures may be followed by death within forty-eight hours, with fibrinous exudate on the peritoneum in which minute grayish nodules are seen. The nodules are made up of a material which is apparently mainly dead or degenerated leucocytes and desquamated peritoneal endothelium, together with many chromatin fragments.

In these acute cases also microscopical examination of the spleen and liver may show the presence of small nodules identical in structure with those seen in the more chronic cases. For the purpose of producing with cultures the characteristic lesions of the testis or its coverings it is better to inoculate the animal subcutaneously, for in the rapidly fatal intraperitoneal inoculations with virulent cultures these may not show any marked changes.

The bacilli may be cultivated from the lesions, but not from the blood of the heart, in the chronic cases. They may be present in the blood of the heart, however, in small numbers in rapidly fatal infections following intraperitoneal inoculation.

Field-mice may die from subcutaneous inoculation in about seventy-two hours. The most conspicuous lesion produced is enlargement of the spleen, with the presence in it of minute grayish nodules. White mice are immune. Rabbits are not so susceptible as guinea-pigs to the infection.

Decolorized by Gram's method. Not motile. Spore-formation not probable. Rate of growth is slow.

Bouillon.—Diffusely clouded, with the formation of a viscid sediment.

Litmus-milk.—Gradually turned red and coagulated.

Agar-agar and Gelatin.—Growth not especially characteristic.

Occurrence.—Found in the lesions of glanders and of farcy, and may invade the blood-stream in small numbers in acute cases of infection. Grows in the tissues in clumps or groups as well as scattered. In lesions on exposed surfaces it may be accompanied by the pyogenic cocci. We have succeeded in demonstrating the presence of the bacillus in the sputum of a case of human glanders by inoculation of a guinea-pig with the sputum.

Diagnosis.—In a case of suspected glanders the discharges from sinuses or ulcerated surfaces, or the contents of pustules, are to be examined for the presence of the bacillus of glanders by the usual methods.

The material for examination may be collected on "swabs." With this a guinea-pig is to be inoculated and cultures and cover-glass preparations are made. If the material be from sinuses or ulcerated surfaces, the isolation of the bacillus by cultures will be difficult, owing to the presence of other organisms. The guinea-pig is to be inoculated in the peritoneal cavity by introducing the infected swab into it through an incision in the abdominal wall, or by injecting about 1 c.c. of a suspension in bouillon of the suspected material into the peritoneal cavity with a hypodermic syringe.

If the bacillus of glanders is present, the scrotum will usually show the characteristic swelling and inflammation in the course of three or four days, and death will occur after some weeks. In some cases the animal may die in thirty-six or forty-eight hours. In any case the characteristic lesions of glanders will be found as described elsewhere, and the bacillus may be isolated from them by cultures. The spleen will practically always yield glanders bacilli in pure culture even if no macroscopic lesion can be made out.

In cultures the organism should show those characteristics of morphology, of culture, and of pathogenesis which have been described above.

Bacillus Proteus (Proteus Vulgaris).—*Morphology.*—Bacilli of very variable length, sometimes appearing like cocci or as filaments.

Motile, being provided with terminal flagella. Does not stain by Gram's method.

Colonies in Gelatin.—Rapid growth with liquefaction of the gelatin. In a medium containing 5 per cent., instead of 10 per cent., of gelatin prolongations from the margins of the colonies may be formed. These may become separated from the mother colonies and form daughter colonies. Motions may be observed in these prolongations.

Gelatin Stab.—Rapid liquefaction along the line of inoculation with cloudiness of the liquefied gelatin and a flocculent deposit.

Agar-agar Slant.—Widely spreading, thin, moist, grayish-white layer.

Potato.—Dirty white, moist layer.

Litmus-milk.—Turned pink and slowly coagulated.

Odor.—The cultures generally have a putrefactive odor.

Pathogenesis.—Intravenous, intraperitoneal, or intramuscular inoculations of rabbits may produce death in twenty-four to thirty-six hours after moderately large doses. Liquefied gelatin-cultures are said to be more virulent than bouillon cultures. Guinea-pigs seem to be less susceptible than rabbits to infection with this organism.

Occurrence.—This bacillus and its varieties are among the most common and widely distributed putrefactive bacteria. It occurs in the intestinal contents. In pathological examinations it may be found in peritonitis and in abscesses, usually associated with other bacteria. It may also invade the circulating blood.

The so-called "proteus group" includes several varieties of similar organisms—viz. the *proteus vulgaris*, the *proteus mirabilis*, and the *proteus Zenkeri*. The latter does not liquefy the gelatin, while the *proteus mirabilis* liquefies it slowly.

Bacillus Mucosus Capsulatus.[1]—*Blood-serum.*—After twenty-four to thirty-six hours in the incubator the colonies appear as translucent, colorless, rounded, convex elevations, resembling drops of mucus. If few in number, they may attain a diameter of 2–3 mm. They are viscid, and when touched with the platinum wire may be drawn out into threads. The water of condensation may become thick or viscid from the growth of the organism in it.

Morphology.—Bacilli of moderate size, usually two or three times as long as broad, with rounded ends, occurring frequently in pairs and sometimes in long forms. Occasionally in cultures it shows a wide capsule. The capsule, however, is best shown in cover-glass preparations from infected tissues (Figs. 78, 80).

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[1] Friedländer: *Fortschritte der Medicin*, 1883, Bd. 1. S. 715; C. Fricke: *Zeitschrift f. Hygiene u. Infectionskrankheiten*, Bd. 23, 1896.

Pathogenesis.—White mice, rabbits, and guinea-pigs die from septicemia in a short time after inoculation, the capsule bacilli being present in the organs and blood of the heart in large numbers.

White mice die in twenty-four hours to three days. Rabbits inoculated in the ear-vein and guinea-pigs inoculated in the peritoneal cavity may die within twenty-four hours.

Subcutaneous inoculation of the animals last named leads only to local suppuration. The lesions produced by this organism consist in marked congestion of the superficial veins, hemorrhage into the lymphatic glands, and enlargement and softening of the spleen. In the guinea-pig a hemorrhagic condition of the supra-renal capsules is present, and in the peritoneal cavity there may be a small amount of clear, rather viscid fluid containing the bacilli in large numbers.

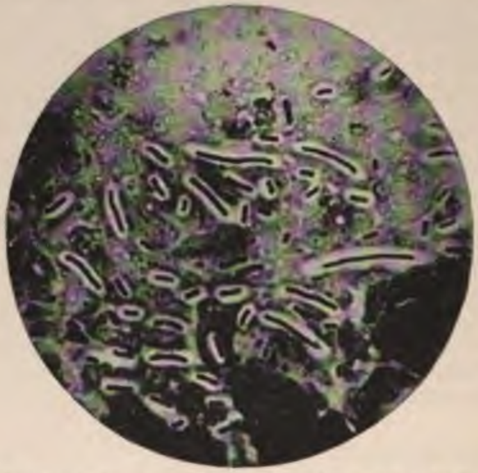

FIG. 78.—Bacillus mucosus capsulatus, from the sputum of a pneumonia patient; × 1000 (Fränkel and Pfeiffer).

The organs on microscopic examination may show peculiar areas in which the cells and nuclei are shrunken and in which the bacilli are aggregated.

Decolorized by Gram's method. Not motile. Does not form spores.

Glucose Agar-agar Stab.—Growth along the line of inoculation, with the production of a few gas-bubbles in the medium.

Bouillon.—Clouded with the formation of a thin pellicle.

Potato.—Thin, colorless, viscid layer.

Litmus-milk.—Turned red and coagulated.

Gelatin.—Growth not remarkable.

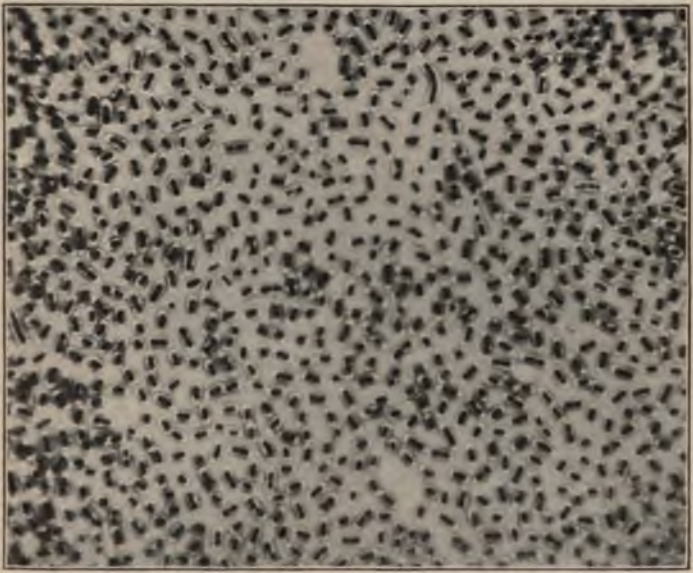

FIG. 79.—Bacillus mucosus capsulatus, from a culture; × 1000 (Wright and Brown).

There apparently exists a number of varieties of aërobic capsulated bacilli differing from one another only in non-essential particulars. The organism here described is to be taken as a type of a group of closely-related bacteria of which the *bacillus pneumoniæ of Friedländer* is a well-known member.

Occurrence.—This organism or closely related forms may be met with in broncho- or lobular pneumonia and in inflammatory conditions of the air-passages generally. It may also be present in the upper air-passages of healthy individuals. It has been observed in inflammations of the middle ear, in empyema, meningitis, endocarditis, peritonitis, and in pus-formations. In fatal infections the blood-stream may be found invaded by the organism. It is held by some bacteriologists that the members of this group may be the infective agents in genuine croupous pneumonia in rare instances. Representatives of this group have been found in the soil, in the air, and in contaminated water.

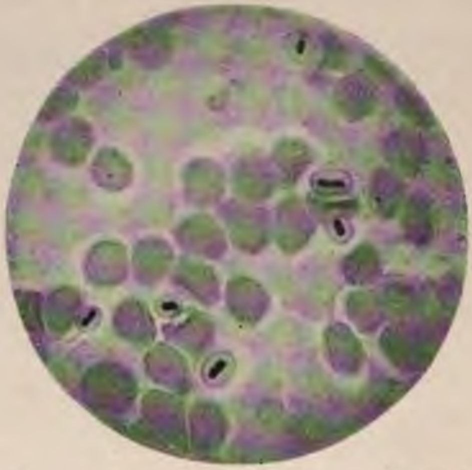

FIG. 80.—Bacillus mucosus capsulatus in blood; × 1000 (Fränkel and Pfeiffer).

Bacillus of Tetanus.[1]—This bacillus will not grow in the presence of oxygen.

Morphology.—Slender rods with rounded ends, which may grow into long threads. In the incubator spores are rapidly formed. These are round, wider than the bacillus, and are situated at the end of the rod, giving the appearance of a drum-stick or a round-headed pin (Fig. 85).

The *colonies* in anaërobic glucose-gelatin cultures appear after several days as small clumps of interlacing fibrillæ from which delicate filaments radiate into the gelatin, which is slowly liquefied.

The colonies in simple anaërobic glucose-agar plate cultures (see page 121) appear, after twenty-four to forty-eight hours in the incubator, as groups and masses of long filaments radiating from a center (Fig. 84).

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[1] Kitasato: *Zeitschrift f. Hygiene u. Infectionskrankheiten*, Bd. 7, 1889.

Pathogenesis.—Subcutaneous inoculation of mice at the root of the tail gives rise to tetanic symptoms in twenty-four hours, followed by death in two or three days.

Guinea-pigs and rabbits are also susceptible to the infection, the period of incubation in these animals being twenty-four to thirty hours in the former and two to three days in the latter animal, after subcutaneous inoculation. The symptoms of tetanus appear first in the extremities nearest the point of inoculation. In mice the hind legs become rigidly extended backward. At the autopsy the bacillus is to be found only at the point of inoculation, and may be difficult or impossible to demonstrate there.

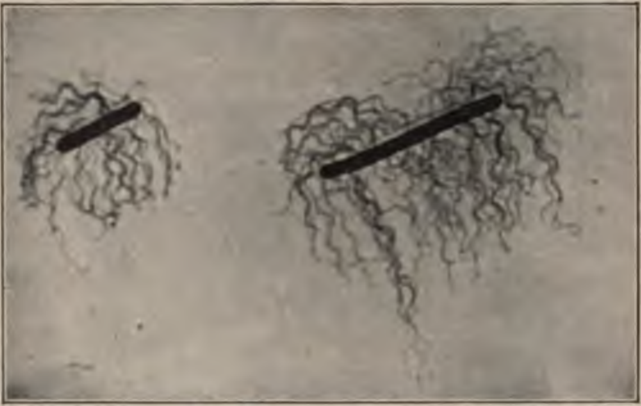

FIG. 81.—Tetanus bacilli showing flagella, from a preparation stained by Dr. Hugh Williams; × 2000 (Wright and Brown).

Glucose-gelatin Stab.—Growth along the line of inoculation, beginning 2 or 3 cm. below the surface, with delicate filaments radiating laterally into the gelatin (Fig. 83). Liquefaction and gas-production occur.

In *deep-stab cultures in glucose-agar* faintly alkaline to litmus (see Fig. 82) growth appears first all along the line of inoculation to within about 1 cm. of the surface after about twenty-four hours in the incubator. Later, lateral outgrowths extend into the medium from all along the line of inoculation below a point about 1 cm. below the surface. In the portion of the line of inoculation above this, growth is frequently observed up to the surface, but without lateral outgrowths. The growth eventually assumes the appearance of an inverted pine tree. A peculiar feature of the culture is the appearance of a brown pigmentation in the culture-medium in its upper layers in the form of a flat or cone-shaped zone. A small quantity of gas may be produced.

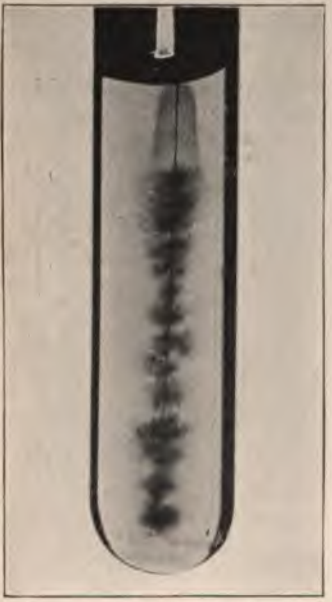

FIG. 82.—Tetanus bacillus. Stab-culture in glucose-agar. In the upper layers of the medium the peculiar brownish coloration is shown.

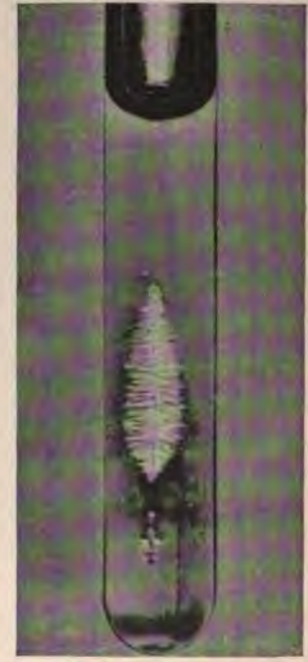

FIG. 83.—Bacillus of tetanus: six-days-old stab-culture in glucose-gelatin (Fränkel and Pfeiffer).

If the agar has a reaction of about 1 per cent. normal acidity to phenolphthalein (see p. 84) growth appears along the line of inoculation and spreads through the medium as a cloudiness extending to within a few millimeters of the surface. The employment of glucose culture-media not older than a week or so seems to be important for success in cultivating this organism.

In the vegetative form the organism is sluggishly motile. It has numerous flagella. It is stained by Gram's method.

Glucose-bouillon.—Growth appears first, after twenty-four to forty-eight hours, as a diffuse cloudiness. Later the fluid becomes clear, and a grayish sediment collects at the bottom of the tube. Only a small amount of gas is produced.

Occurrence.—Found in the soil, and often in the feces of herbivorous animals. In cases of tetanus the bacillus is to be found only in the wound or at the point of inoculation. It does not invade the blood-current.

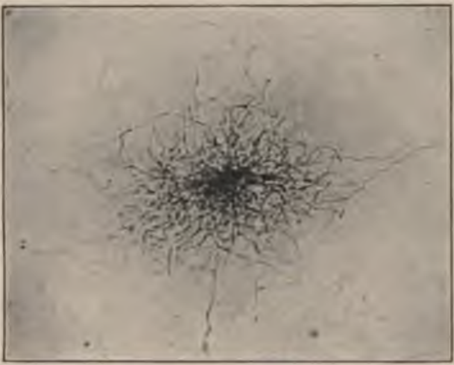

FIG. 84.—Colony of tetanus bacilli in anaërobic glucose-agar plate; low magnifying power (Wright and Brown).

The bacillus of tetanus acts by the production of a "*toxin*" or "*toxalbumin*." This is also produced in cultures. It may be demonstrated in the bacteria-free filtrate of bouillon cultures some days or weeks old. A very few drops of this fluid will give rise to fatal tetanus in a mouse.

Method of Isolation.—Tetanus bacilli will grow in aërobic culture if other bacteria are growing with them. Since tetanus wounds usually contain other bacteria, all that is necessary to obtain an impure culture of the tetanus bacillus is to inoculate an ordinary blood-serum culture-tube (see page 79) with material from the wound. After several days or a week in the incubator, if tetanus bacilli are present they can be recognized by cover-glass preparations from the growth in the tube by their morphology and spore-formation (see Fig. 85). There will also be a peculiar, stinking odor about the culture. The isolation of the tetanus bacillus is now to be proceeded with as follows: Mix a loopful of the mixed growth on blood-serum with a tube of sterile bouillon, and heat in a water-bath for at least fifteen minutes at 80° C., then make anaërobic cultures from this (see Anaërobic Methods, page 119), taking several loopfuls for inoculation.

If other spore-bearing bacilli are present in the mixed culture in the blood-serum tube, it will be necessary to use some form of anaërobic culture on a solid medium in order to obtain separate colonies of the tetanus bacillus for further cultures.

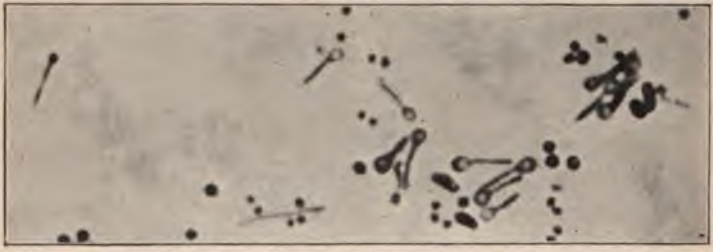

FIG. 85.—Spore-bearing tetanus bacilli in an impure culture on blood-serum from a case of tetanus. In the bacillus on the extreme left the beginning of spore-formation is shown (Wright and Brown).

The bacillus may be isolated from wounds and from the soil by inoculation of mice subcutaneously, and proceeding as above described with material from the seat of inoculation.

Bacillus Aërogenes Capsulatus.[1]—Will not grow in the presence of oxygen.

Morphology.—Bacilli of about the thickness of the anthrax bacillus, variable in length, but usually 3 to 6μ long. Ends rounded or square cut. Occurs singly, in pairs, in clumps, and sometimes in short chains, less frequently in threads and long chains.

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[1] Welch and Flexner: *Journal of Experim. Medicine*, vol. i. No. 1, 1896.

May show unstained spots or deeply staining granules in the protoplasm. Capsules may be frequently demonstrated in the specimens from the tissues, and sometimes in agar-agar cultures.

Colonies in anaërobic cultures are grayish to brownish-white, with a central darker spot by transmitted light. In time they may attain a diameter of 2 to 3 mm. or more. Colonies in the depths are spherical or oval, sometimes presenting knob-like or feathery projections.

Effects on Animal Tissues.—Not pathogenic for rabbits.

If a rabbit that has received 0.5 to 1 c.c. of a bouillon culture injected into the ear-vein be killed immediately afterward and the body kept for twenty-four hours at a temperature of 18° to 20° C., or for four to six hours at a temperature of 30° to 35° C., the vessels and organs will be found to contain a great quantity of gas and large numbers of the bacilli. The organism multiplies post-mortem in the blood of the animal and produces the gas. This effect upon the tissues of the dead animal is characteristic of the bacillus.

The subcutaneous inoculation of guinea-pigs with young cultures may produce fatal gas phlegmons. The hemorrhagic fluid from the dead animal is virulent for other guinea-pigs, and may be virulent for rabbits.

Gas-production is marked in agar-agar and gelatin cultures containing glucose. The gas produced burns with a blue flame and is odorless.

Gelatin is liquefied slowly and to a limited extent.

Glucose Bouillon.—Diffusely clouded at first, later becoming clearer, with an abundant whitish, more or less viscid sediment.

Milk.—Coagulated, the clot being firm, retracted, and furrowed with the marks of gas-bubbles.

Potato.—Growth thin, moist, and grayish-white, or it may not be visible.

The bacillus is stained by Gram's method. It is not motile. Spore-formation has been observed by E. K. Dunham.

The vitality of the organism depends upon the character of the culture-medium and the mode of cultivation. It survives longer when cultivated by Buchner's method (see page 122) than when cultivated under hydrogen. Cultures on glucose media are shorter lived than those on plain media.

Occurrence.—Occurs at autopsies in which gas-bubbles are present in the larger vessels, accompanied by the formation of numerous small cavities in the liver containing gas. It has been found also in emphysematous phlegmons, in puerperal sepsis, in peritonitis, and in other conditions.

Bacillus of Malignant Edema.[1]—This bacillus will not grow in the presence of oxygen.

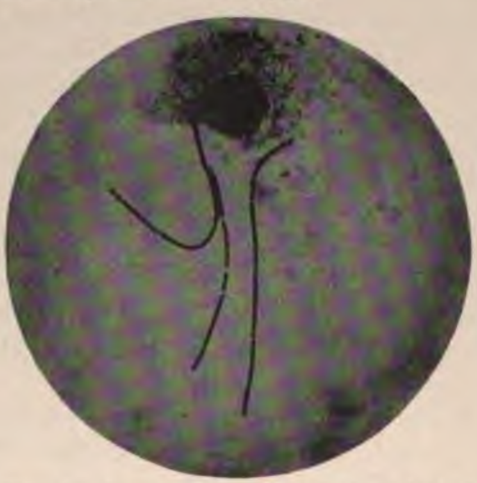

FIG. 86.—Bacillus of malignant edema from the edema fluid of a guinea-pig inoculated with garden-earth; × 1000 (Fränkel and Pfeiffer).

Morphology.—Rather large bacilli, sometimes growing into threads (Fig. 86), but occurring frequently in pairs, in which the proximal ends are square while the distal ends are rounded. Forms oval spores in the middle of the rod, which may give the rod a spindle or oval shape.

The *colonies* in anaërobic glucose-gelatin cultures appear as spheres of cloudy liquefied gelatin marked by delicate radiating streaks. Gas-bubbles are formed in the medium (Fig. 87).

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[1] Liborius: *Zeitschrift f. Hygiene u. Infectionskrankheiten*, Bd. 1, 1886.

Pathogenesis.—Subcutaneous inoculation of mice, guinea-pigs, and rabbits is followed by death in from sixteen to forty-eight hours, depending upon the animal, mice being most susceptible. The typical lesions are extensive subcutaneous edema containing gas-bubbles and more or less blood, and enlargement of the spleen. The bacilli are found in the edema, in the viscera, and on the serous surfaces of the organs, but not in the blood of the heart if the examination be made immediately after death, except sometimes in mice. The organism is not capable of multiplying in the living blood, owing to the presence of oxygen. In inoculating subcutaneously a deep pocket should be made in the skin, and the material for inoculation introduced into the tissue as far away from the opening as possible. This is to prevent the access of too much oxygen to the organism.

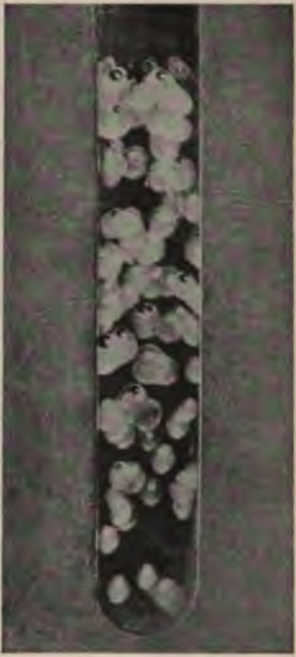

FIG. 87.—Bacillus of malignant edema; colonies growing in glucose-gelatin (Fränkel and Pfeiffer).

Slightly motile. Flagella may be demonstrated by special staining methods.

Decolorized by Gram's method.

Growth in anaërobic agar-agar and bouilion culture is good, but not characteristic.

Occurrence.—Widely distributed in the soil and in putrefying substances. Only a very few cases are on record of infection in man by this bacillus.

Actinomyces.—The proper classification of the micro-organism known as actinomyces is somewhat uncertain. The published descriptions of various examples of it vary widely, and have led to much confusion.

It is probable that some of the micro-organisms cultivated and described as actinomyces were not such, but were contaminations or secondary invaders of the tissues.

Probably the best opinion to-day considers actinomyces to belong to a group of micro-organisms which, on account of their branching, are regarded as more highly developed than are the bacteria, but which are not so highly organized as are the moulds or hyphomycetes. The members of this group, of which a number are known, are named streptothrices. While there is some objection to this name, it seems to us to be satisfactory.

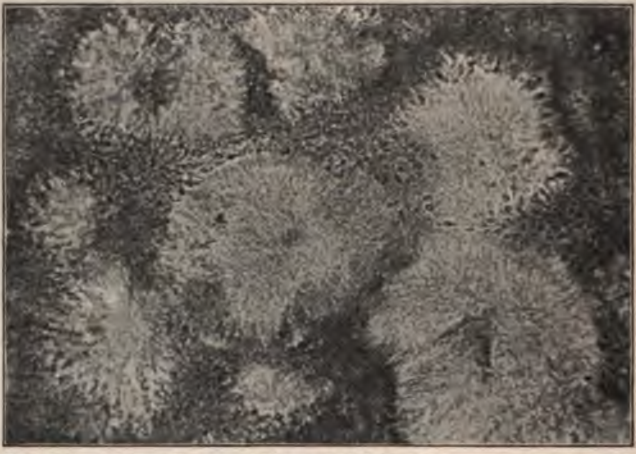

FIG. 88.—Actinomyces granule crushed beneath a cover-glass, showing radial striations in the hyaline masses. Preparation not stained; low magnifying power (Wright and Brown).

The organism appears in the pus from subacute or chronic suppurative lesions of the disease actinomycosis, as grayish or yellowish granules, usually less than 1 mm. in diameter. Sometimes these granules are aggregated in groups of two or three, and thus appear as lobulated larger granules. They are friable, and when gently crushed beneath a cover-glass and observed under the microscope, they are seen to have been broken up into hyaline rounded masses, at the margins of which, on close inspection, fine radial striations or filaments or hyaline club-shaped bodies, all closely set together, may be seen (Figs. 88, 89). The club-shaped bodies are variable in size, and are composed of a hyaline, refringent substance. The appearance of radial striation in the granule, when observed with the microscope, due to the presence and radial arrangement of these hyaline bodies, gave rise to the name "ray-fungus" for this parasite. Not all of the granules have these "clubs." In the granules obtained from the lesions in man they are much less frequently observed than in those obtained from the lesions in cattle.

If a cover-glass preparation be made by breaking up one of the granules and staining with Gram's method, there will usually be found, upon examination with an oil-immersion lens, isolated and matted filaments, many of which may be seen to branch, in addition to longer and shorter fragments of filaments and fine detritus of the same (Fig. 90). The filaments are usually more or less wavy in their course, and are, as a rule, slightly thicker than the tubercle bacillus. Some of the filaments will be found to stain homogeneously; others do not stain so deeply, and show numerous deeply staining points in their substance. If clubs are present in the granule, they also may be found scattered throughout the preparation.

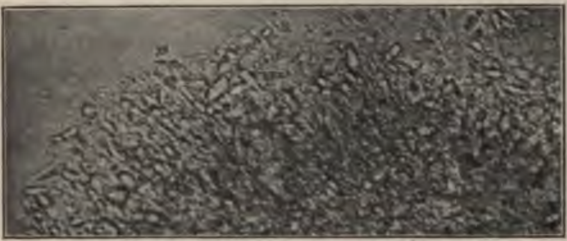

FIG. 89.—A portion of an actinomyces granule crushed beneath a cover-glass, showing the "clubs." The preparation not stained; moderately high magnifying power (Wright and Brown).

In sections of the tissues stained by Gram's method two chief forms of granules are found. In one of these forms the granule is seen to consist of filaments embedded in a hyaline substance, and usually arranged at the periphery in an indefinite radiate manner (Fig. 91). At the margin of the granule the filaments are usually much more numerous than in the central portions, where the hyaline material predominates. This hyaline material apparently consists of degenerate or dead filaments or their remains. The other form of granule seen in sections is distinguished by possessing at its margin a row of closely set radiating club-shaped bodies composed of hyaline substance which does not stain by Gram's method (Fig. 92). These are the "clubs" previously mentioned, and they may occupy more or less of the circumference of the granule. In certain instances a Gram-staining filament may be seen in the central portion of a club. The main mass of this form of granule is not essentially different from that of the first-mentioned form. The characteristics of both forms of granule may be found in some granules.

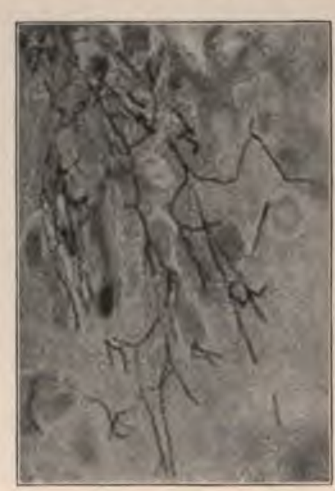

FIG. 90.—Branching actinomyces filaments in a cover-glass preparation made from an actinomyces granule stained by Gram's method; × 1000 (Wright and Brown).

The club-shaped bodies are to be regarded as products of degeneration of the marginal filaments.

In some cases isolated or small groups of filaments may be found scattered among the pus-cells in the lesions.

Cultures.—Actinomyces grows best in the incubator. On the surface of slant tubes of blood-serum and agar-agar the organism grows in larger and smaller grayish-white, shining, elevated colonies. If the colonies are few in number, they may attain a diameter of several millimeters, with highly elevated, irregular central portions and thin, wavy, marginal portions. If the colonies are very numerous, they may be confluent, and form a grayish granular layer. Growth on the surface takes place more rapidly, and with a development of more numerous colonies if the culture be kept under anaërobic conditions, the maximum of growth being obtained in about a week.

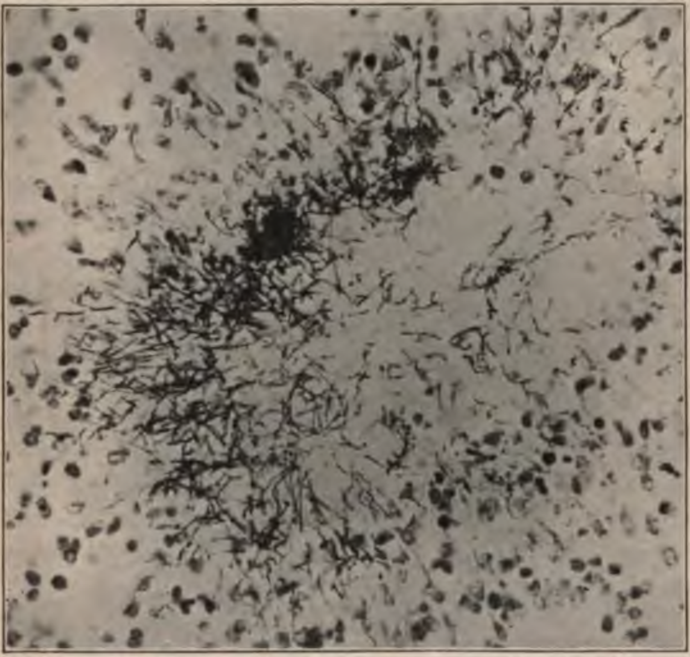

FIG. 91.—Colony or granule of actinomyces in a section through a lesion, showing the Gram-stained filaments and hyaline material and also the pus-cells surrounding the colony (Wright and Brown).

In stab-cultures in sugar-agar the colonies appear as grayish granules along the line of inoculation.

In bouillon the organism grows in the form of grayish-white, friable granules or clumps at the bottom of the tube.

The maximum of growth is obtained in about four or five days under aërobic conditions. The culture-fluid is never clouded.

The morphology of the organism in cultures is subject to considerable variation.

In bouillon-cultures it grows in the form of longer or shorter filaments which sometimes may be seen to branch. Scattered along the filaments are sharply defined, deeply staining, oval or rounded areas occupying the whole diameter of a filament. Clubs are never observed in cultures, but sometimes round or oval swellings may be seen at the ends of the filaments in the older cultures.

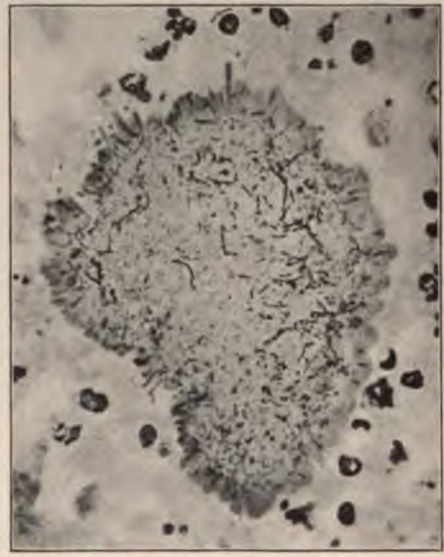

FIG. 92.—Colony or granule of actinomyces in a section through a lesion, showing the peripheral arrangement of the "clubs." In several instances the central stained filaments in the "clubs" are seen; × 750 (Wright and Brown).

In cover-glass preparations from cultures on solid media the organism appears as longer and shorter rods, often more or less fusiform or club-shaped, and as slightly wavy filaments of variable length, sometimes segmented.

Diagnosis.—The finding of the granules in suspected pus may be facilitated by spreading the pus on a slide.

The identification of the organism is made certain only when the granules have been found to present the appearances described above after crushing under a cover-glass, and after cover-glass preparations made from them and stained by Gram's method show the branching filaments.

Mycetoma (Madura Foot).—Mycetoma is a chronic inflammatory process, most commonly affecting the tissues of the foot, in which suppurative nodular swellings, sinus-formation, and enlargement and distortion of the parts are prominent features. The bones may become involved and undergo a rarefying osteitis.

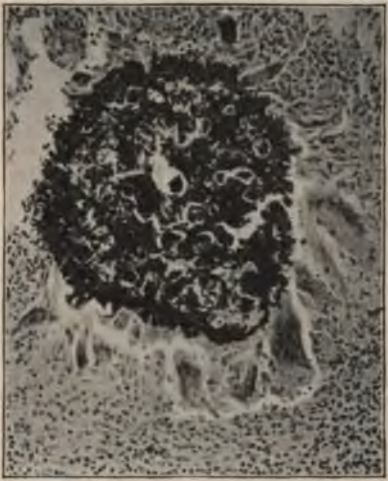

FIG. 93.—Mycetoma. Black granule in a section from the lesions. The black rounded central mass represents the brownish hyaline refringent substance in which the fungus elements are embedded. Giant cells and granulation-tissue are shown (Wright and Brown).

The affection is characterized by the presence in the diseased tissue and in the discharge from sinuses, of peculiar granules, usually not more than one millimeter in diameter, but sometimes larger. These granules in certain cases are of a black color, of irregular shape, and are hard and rather brittle. In general they resemble grains of gunpowder. In other cases the granules are whitish, grayish, or yellowish in color, and are of a soft or cheesy consistency. They have been compared to fish-roe in appearance. A very few cases are also recorded in which the granules were of a red color. There is no good evidence to show that more than one kind of granules are ever present in any one case of the disease.

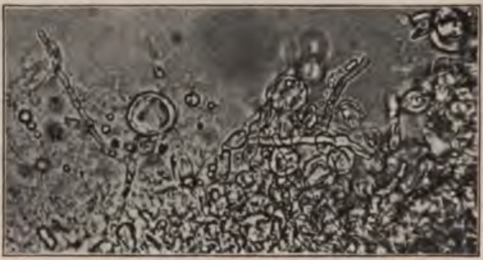

FIG. 94.—Mycetoma: fungus elements from a black granule after maceration; high magnifying power (Wright and Brown).

On account of the fact that at least two very different kinds of granules are found associated with the lesions, two varieties or forms of the disease are recognized: the "melanoid" or black variety, in which the granules are black, and the "ochroid" or pale variety, in which the granules are white to yellow in color.

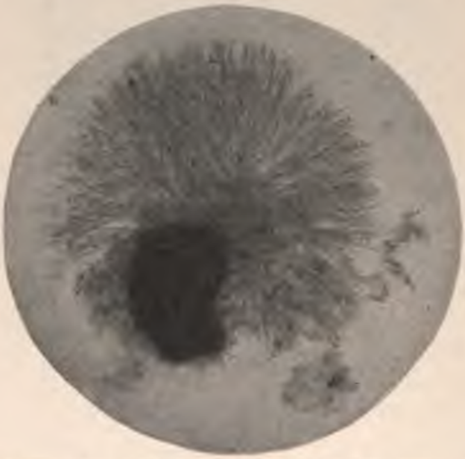

FIG. 95.—Mycetoma, showing the outgrowth of hyphæ from one of the black granules after planting in fluid culture-medium (Wright and Brown).

The pale variety is probably actinomycosis of the part, the "melanoid" or black variety is clearly not actinomycosis, and the parasite belongs to the hyphomycetes, a very different class of organisms from that to which actinomyces belongs.

The disease occurs frequently in India and some other sub-tropical countries. It is extremely rare in temperate climates.

Wright has studied a case of the black variety of the disease, and upon the results of this study[1] the following description of the parasite is based:

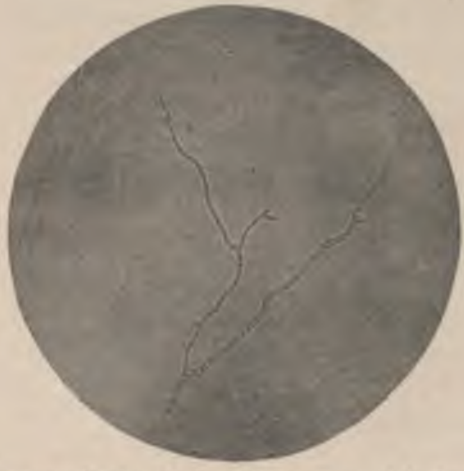

FIG. 96.—Mycetoma, showing details of the structure of the hyphæ developed from the black granules (Wright and Brown).

The black granules, characteristic of this variety of the disease, consist of a hard, rather brittle, amorphous, brownish or black substance in which a fungus mycelium is embedded (Fig. 93). These fungus elements may be demonstrated by macerating the granules in a strong solution of alkali or sodium hyposulphite. This disintegrates the granule and dissolves the brown embedding substance. The fungus elements obtained from the granules by this maceration are shown in Fig. 94.

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[1] *Journal of Experim. Medicine*, 1898, vol. iii. Nos. 4 and 5.

The organism grows on ordinary culture-media about as rapidly as do the ordinary moulds of the laboratory.

From a granule planted in bouillon numerous hyphæ grow out into the fluid and may form eventually a whitish powder-puff-ball-like structure with the granule in the center (Fig. 95). The hyphæ grow very long, are septate, have in places vacuoles, and form branches (see Fig. 96); on the surface of agar-agar the growth appears as a meshwork of widely spreading filaments of a grayish color.

On potato it forms a dense, widely spreading, coherent membrane of velvety surface. In its central portions it is of a pale-brown color, but at the periphery it is white. A marked feature is the appearance of small spherical droplets of coffee-colored fluid on the surface of the growth after a time.

It must be quite clear from the foregoing that the organism belongs to the moulds or hyphomycetes.

The formation of reproductive organs, such as spore-bearing hyphæ, was not observed.

The results of animal inoculations were negative.

Rabies (Hydrophobia).—The diagnosis of this disease from a pathological standpoint is usually made by the production of experimental rabies in a rabbit by intradural inoculation with material from the nervous system of the animal suspected to have died of it. The poison of the disease is found in the brain, spinal cord, salivary glands, and pancreas. For purposes of inoculation a piece (1 or 2 c.c.) of the medulla or brain, preferably the former, is rubbed up in a sterilized mortar with about 10 c.c. of sterilized distilled water. The resulting fluid is filtered through absorbent cotton, and then through filter-paper, to remove tissue-shreds. Of the clear fluid thus obtained 4 or 5 drops are injected beneath the dura of a rabbit by means of a hypodermic syringe, the skull being trephined with a small trephine about 4 mm. in diameter. The most favorable place for opening the skull is at a point in the median line just posterior to a line drawn through the middle of each eye.

The symptoms of experimental rabies in the rabbit first manifest themselves after two weeks, never earlier, but they may not appear until later, not even until two months have passed. The first symptom is a weakness of the hind legs, followed by paralysis. The paretic condition soon extends to the fore legs, dyspnea appears, and death usually occurs in about three days after the onset of the symptoms. Paralytic symptoms developing before two weeks are not due to infection with rabies, but to some other cause; for instance, infection with the pneumococcus or other bacteria which may be present in the material inoculated.

During the course of the disease the animal never appears stupid, with dull eyes, as in other infections, but remains "conscious," so to speak, until the last.

PART III.

HISTOLOGICAL METHODS.

Introduction.—The ideal function of the technique of pathological histology is so to fix tissues for microscopic examination that every tissue-element or pathological product is perfectly preserved with all its morphological and chemical properties intact, and so to stain tissues that every tissue-element or pathological product can be readily differentiated from any other tissue-element or pathological product that resembles it. In certain respects only has this ideal yet been reached, but the number of differential stains is increasing yearly.

In the following pages the various steps in the preparation and staining of tissues have been arranged, so far as possible, in logical sequence.

LABORATORY OUTFIT.

Microscopes.—The most important laboratory instrument is the microscope. It should be, so far as means will permit, the best that skill can produce. The two foreign makes of microscopes most in favor in this country are the Zeiss and the Leitz. Excellent microscopes are also made by Reichart, Hartnack, and Seibert. American microscopes have greatly improved during the past few years, but do not yet reach the standard set by the best foreign makes. Undoubtedly the best microscopes in every particular and the most expensive are those manufactured by Zeiss.

It is important for a beginner in microscopy, before buying a microscope of any make, to have it carefully examined and its lenses tested at a pathological or other laboratory by some one skilled in its use. The continental form of stand of medium size is to be preferred to all others. The large stand is undesirable, because it is too heavy and too high for comfortable use. It should be furnished with rack and pinion, and with micrometer screw for coarse and fine adjustment, with a triple nose-piece, and with an Abbe condenser and iris diaphragm. The necessary objectives are a low and a high dry, and a $\frac{1}{12}$ oil-immersion. Two eye-pieces, a low and a high, will be found sufficient for all ordinary purposes.

The stands, oculars, and objectives generally used are the following:

Zeiss: Stands, II*a*, IV*a*, IV*b*.
Oculars, 2 and 4.
Objectives, AA, D, and $\frac{1}{12}$ oil-immersion.
Leitz: Stands, I*a*, I*b*, II, II*c*.
Oculars, 1 and 3.
Objectives, 3, 7, and $\frac{1}{12}$ oil-immersion.

Even if all these different parts cannot be purchased at the same time, it is important to buy a stand to which they afterward may be added, for the list includes only what every medical practitioner should have at his service for the proper examination of urine, sputum, blood, etc.

The apochromatic lenses and compensation oculars are too expensive to come into general use. Fortunately, they are more important for photomicrography than for general microscopic work.

The *oil-immersion lens* should always be cleaned after using by wiping off the oil with an old linen or silk handkerchief or with the fine tissue-paper now manufactured for that purpose. If the lens is sticky, moisten the cloth with benzol or xylol; Leitz recommends alcohol. The same process can be used if necessary for the dry lenses, but it must be done quickly, so as not to soften the balsam in which the lenses are imbedded. Ordinarily a dry cloth is sufficient.

In using the *Abbe illuminating apparatus* it is important to bear in mind that the best results are obtained, according to Zeiss, by employing the plain mirror, for the condenser is designed for parallel rays of light. The concave mirror is to be used only when some near object, such as the window-frame, is reflected into the field of vision or when artificial light is employed.

A *mechanical stage* is now made which can be instantly attached to any microscope. It is exceedingly useful for blood-counting or for searching carefully the whole surface of a stained cover-slip. For ordinary work it is undesirable.

For microscopic work the best *illumination* is that obtained from a white cloud. When artificial light is necessary, the Welsbach burner, or, better still, the Edison electric lamp with ground-glass globe, will be found very satisfactory. The slight yellowish tint of the light can be corrected, if necessary, by means of a piece of blue glass inserted over the mirror or just below the object to be examined.

For drawing, the *Abbe camera lucida* will be found extremely useful and convenient. Leitz has recently constructed two new drawing oculars, of which the one to be used with the microscope inclined at an angle of 45° would seem to be very practicable. With the other the drawing surface has to be inclined at an angle of 12° to avoid distortion of the image.

Freezing Microtome.—Freezing by means of the evaporation of ether, more rarely of rhigolene, is the method in general use. The process is both expensive and slow. A much cheaper and more rapid method of freezing was originated several years ago in the Sears Pathological Laboratory by Dr. S. J. Mixter, and has since been in constant use both here and in the hospitals in Boston. This method consists in the employment of compressed carbon-dioxid, which is found in commerce in iron cylinders containing each about twenty pounds of liquefied gas. It is commonly used for charging beer and soda-water. As a rule, the cylinders are loaned, so that it is necessary to pay for the contents only.

In the original design the microtome was connected with the cylinder by means of a rubber tube. Recently Dr. C. R. Bardeen has introduced a great improvement by modifying the microtome so that it can be connected directly with the cylinder; he has also inserted a second valve, which affords greater control over the escape of the gas (Fig. 97).

In freezing, the valve should be turned carefully, so that the gas may escape slowly and evenly. Pieces of tissue can easily be frozen in a few seconds, but it is much better to freeze the tissue neither too rapidly nor too hard, otherwise the sections will show on microscopic examination multiple parallel lines of fracture due to their being bent by the knife while still brittle. Accordingly, when the tissue is frozen too hard, it is best to wait until it thaws a little, or else to soften the surface each time just before cutting a section by rubbing over it the tip of a finger moistened in water or salt solution. Pieces of tissue for freezing should not be over 2 to 4 mm. thick. Alcohol specimens must first be thoroughly freed from spirit by soaking in running water for some time, usually several hours.

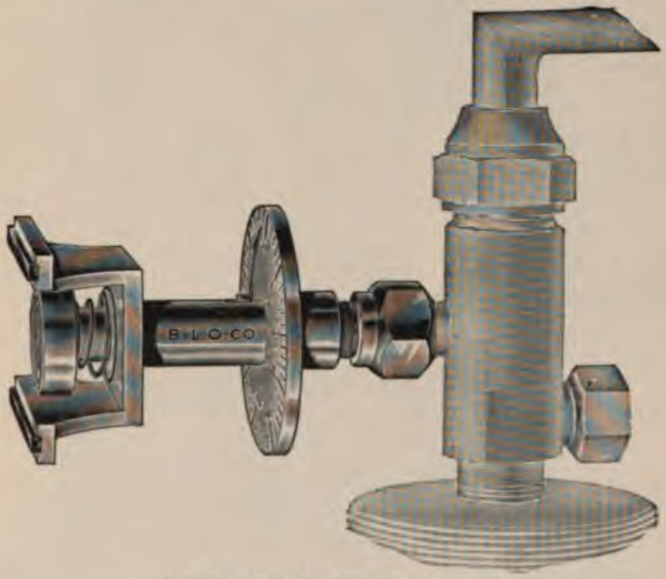

FIG. 97.—Bardeen freezing microtome.

For cutting frozen sections the blade of a carpenter's plane mounted in a wooden handle (Fig. 98) will be found very serviceable and easy to sharpen.

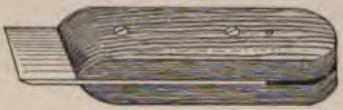

FIG. 98.—Knife for freezing microtome, made from the blade of a carpenter's plane.

Celloidin Microtome.—There are two types of celloidin microtomes—one in which the object is raised by a screw, a second in which the object is raised by being moved up an inclined plane. The first type of machine is the better, for two reasons: the screw affords greater accuracy in the even elevation of the object than is possible with an inclined plane, and the object remains at all times in the same relative position with regard to the knife, so that an equally long sweep of the blade can be obtained for every section. An excellent instrument of this type is made by Bausch & Lomb (Fig. 99). For practical work it is much to be preferred to

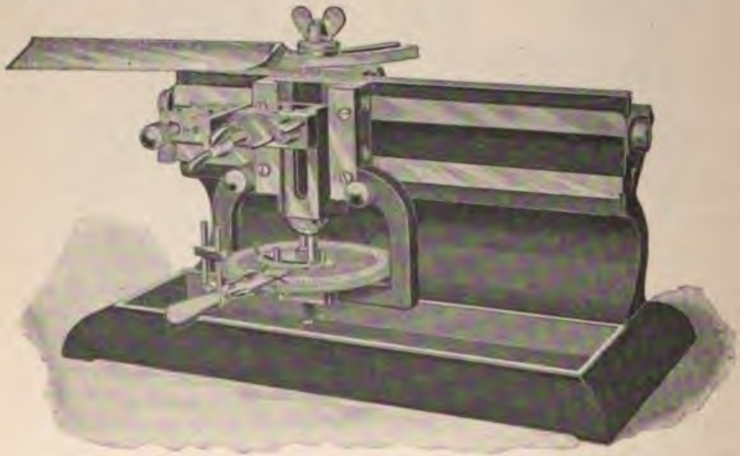

FIG. 99.—Large laboratory microtome.

the elaborate Schiefferdecker-Becker microtome, designed for cutting sections under alcohol.

A new and wholly original microtome, in which the knife remains fixed and is clamped at both ends, while the object-holder, which is raised by a screw, moves back and forth beneath the knife, has recently been designed by Dr. C. S. Minot and is manufactured by Bausch & Lomb (Fig. 100). It is intended both for celloidin and for paraffin work. When but

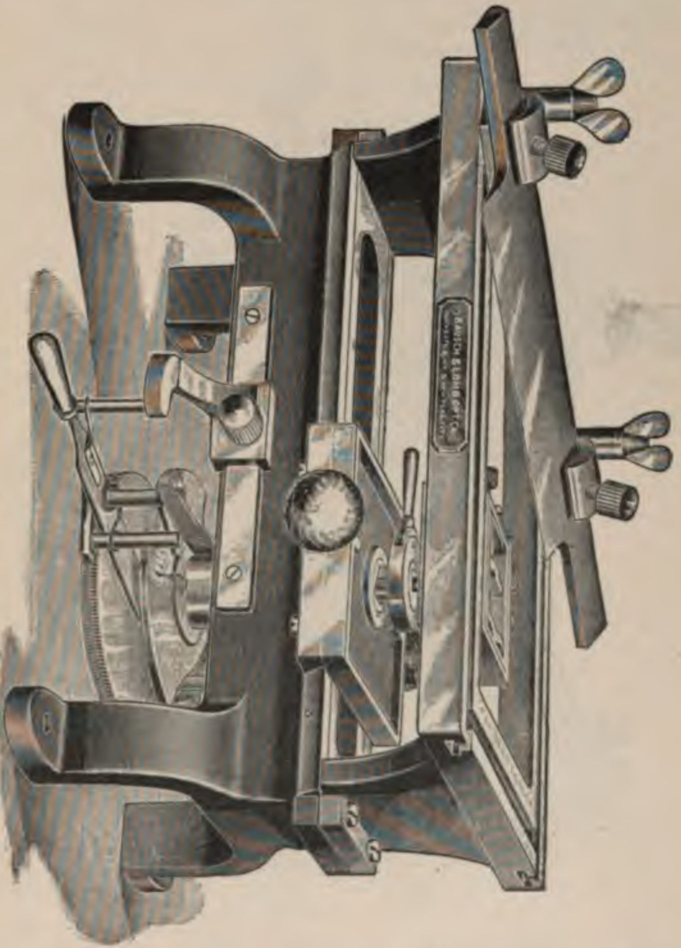

FIG. 100.—Minot's precision microtome.

one instrument can be afforded, it is believed that this model will be found the most serviceable for both kinds of work.

A *drop-bottle* on an elevated stand, with screw arrangement for regulating the amount of alcohol, is the most convenient method for keeping the object and the knife wet while cutting; 80 per cent. alcohol should be used.

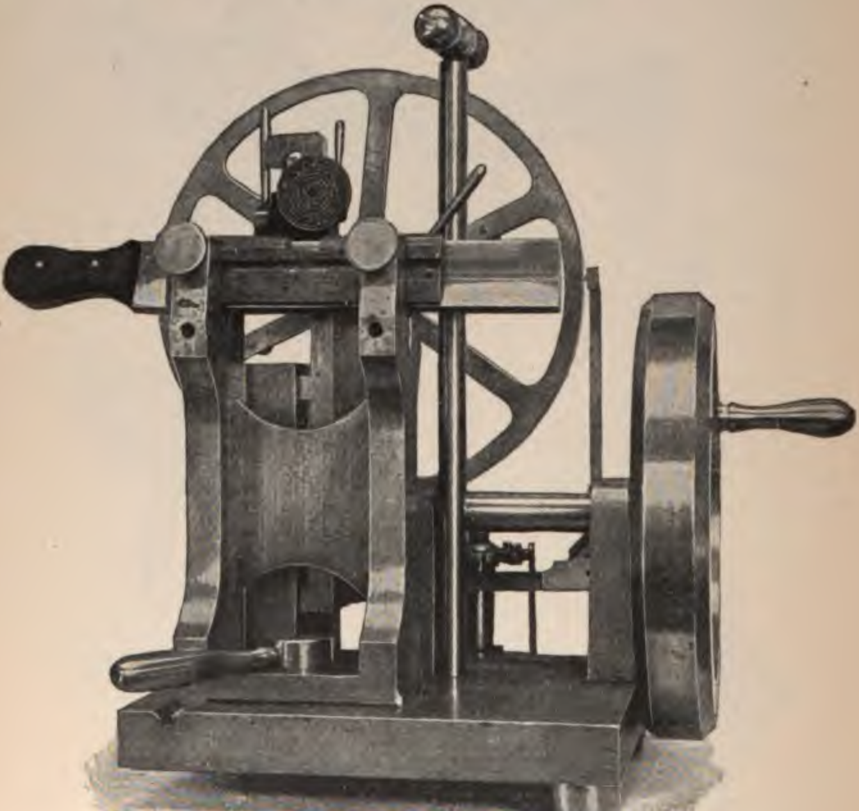

FIG. 101.—Minot-Blake microtome.

The Minot-Blake Microtome.[1]—This microtome is specially designed for cutting sections of 1 and 2 micra in thickness, but it may be adjusted to cut sections up to 10 micra. The main sliding parts are designed to take advantage of the principle of three-point bearings, so as to avoid minute irregularities in their motions. The knife is provided with a brace to prevent the elasticity of the knife-edge from causing "skipping" when the machine is adjusted to 1 or 2 micra.

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[1] This microtome is made by the Buff Manufacturing Co., 505 Atlantic Ave., Boston, Mass.

In operating this machine for thin sections the following important points should be borne in mind:

1. The machine must be in accurate adjustment. The brace to the knife is to be so applied as not to produce a twisting strain upon it. The upper edge of the brace should lie about $\frac{1}{50}$ of an inch below the knife-edge. The knife is to be canted just enough to obtain clearance, and no more.

2. The knife-edge should be so well polished that, when observed with the microscope under a magnifying power of about 90 diameters, it appears as a straight line.

3. The specimen must be well embedded, and the paraffin block containing it, as well as the brass carrier to which it is attached, should be cooled in ice-water immediately before cutting. The knife and its brace should be likewise cooled. The paraffin block is to be carefully trimmed as described elsewhere.

Paraffin Microtome.—Although paraffin sections can be cut on a celloidin microtome, it is preferable to have an instrument designed for the purpose. The Minot wheel microtome manufactured by Bausch & Lomb (Fig. 102) can be thoroughly recommended.

Paraffin Bath.—The best bath for keeping paraffin at a constant temperature is a thermostat of suitable size with hot-water jacket, such as is used for growing cultures of bacteria. The paraffin is kept in it on shelves in glass dishes of various sizes. The advantages of this method over the old way of using copper cups set into the top of a water-bath are that the paraffin is kept absolutely free from dust, each worker can have his own set of dishes, and the smallest bits of tissue can be readily found in them, because they are transparent.

A paraffin melting at 50° to 52° C. will be found convenient for use throughout the year if the laboratory is kept fairly warm in winter. Many workers, however, prefer one paraffin melting at 45° C. for winter, and another melting at 48° C. for summer.

A preliminary bath of soft paraffin is wholly unnecessary, and only prolongs the objectionable stage of heating. The regulator should register only one or two degrees above the melting-point of the paraffin.

Paraffin should be melted and decanted or filtered before use, as it often contains foreign material. When hot it runs through an ordinary filter without trouble. A hot-water jacket to the funnel is not at all necessary.

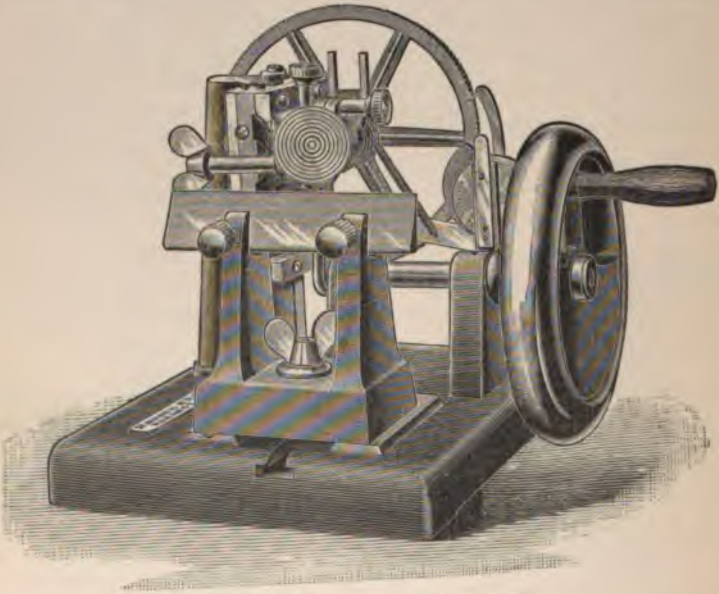


FIG. 102.—Minot wheel microtome.

Vulcanized Fiber.—For mounting celloidin preparations nothing is so poor as cork, although it has been in use for years. The chief objections to it are that it does not furnish a rigid support to the imbedded object; that, unless weighted, it floats in alcohol with the specimen downward; and that it yields a coloring material which stains both the alcohol and the specimen. Wood is not much better, although, of course, much firmer. Glass blocks have been proposed, and might do fairly well if there did not exist an ideal substance—viz. vulcanized fiber. This can be obtained in boards or strips, preferably $\frac{1}{2}$ or $\frac{5}{8}$ inch in thickness and sawn to any desired dimensions. It is perfectly rigid, is heavy enough to sink specimens to the bottom of the jar in an upright position, is unaffected by alcohol or water, is light red in color, so that it is easily written on with a lead pencil, gives off no coloring material, and is practically indestructible.

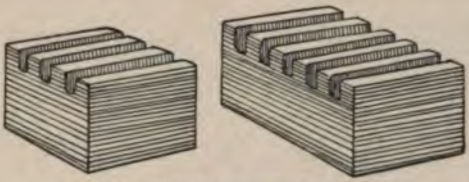


FIG. 103.—Blocks of vulcanized fiber.

Two or three parallel cuts, 1 to 2 mm. in depth, should be sawn into the upper surface of each block, so as to give the celloidin a firm hold.

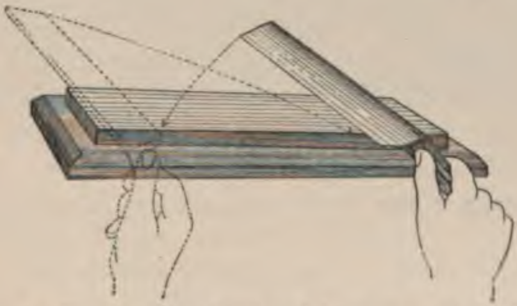


FIG. 104.—Diagram of the direction of the movements in honing.

Knives.—The knives for both the celloidin and the paraffin microtomes should be heavy and not too long, so as to afford as great rigidity as possible; they should be biconcave, so that they may be easily sharpened. It is important that every one who does much work in a pathological laboratory should learn to sharpen his own knives. The requisite skill is not difficult to acquire, and the time spent in learning is fully compensated for by the ability always to have a sharp knife when it is wanted. For honing a knife either a fine water-stone or a glass plate with diamantine and Vienna chalk may be used. In honing, the edge of the knife is forward and the motion is from heel to toe. The knife should always be turned on its back, and the pressure on it should be at all times rather light.

In stropping, the movement is reversed. The back of the knife necessarily precedes the edge, and the motion is from toe to heel. The direction of the movements in honing and stropping is best illustrated by the diagrams (Figs. 104, 105).

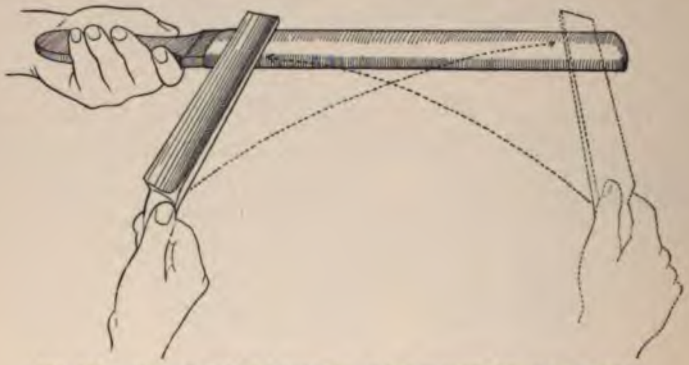


FIG. 105.—Diagram of the direction of the movements in stropping.

Running water for washing out specimens which have been fixed in Flemming and other solutions is most easily supplied by having a water-pipe, furnished with numerous cocks 5–10 cm. apart, run horizontally over a slightly sloping shelf adjoining the sink. Attached to each cock is a rubber tube, with a glass tube in the end of it long enough to reach to the bottom of the jar (Fig. 106). By this arrangement the amount of water supplied to each specimen can be easily regulated.

Slides should be of colorless glass with ground edges. The English form, measuring 1 × 3 inches (76 × 26 mm.), is to be preferred for ordinary use. Occasionally broader slides are needed.

Cover-slips should be square or oblong according to the shape of the specimen. Most dry lenses are adjusted for cover-glasses measuring 16 or 17μ in thickness, so that if possible no cover-slips ranging outside of 15 to 18μ should be used. With an oil-immersion the exact thickness is not quite so important.

Slides and cover-slips are cleaned by dipping in alcohol and wiping dry with a soft crash towel or old linen handkerchief.

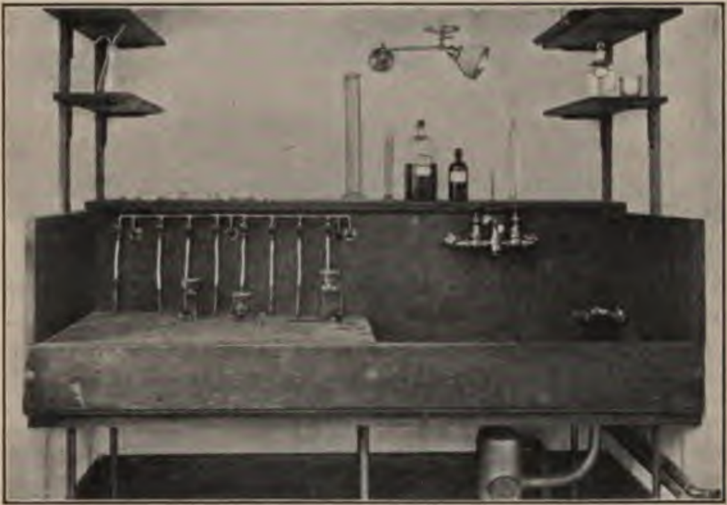


FIG. 106.—Large laboratory sink, showing adjoining shelf and arrangement for running water.

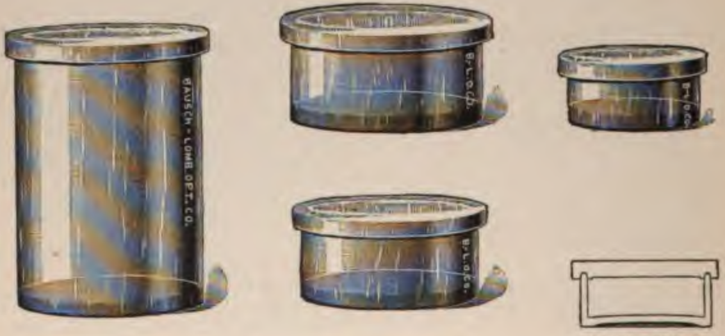
To clean old slide preparations, heat them until the balsam softens so that cover-slips and slides can be drawn apart. The slides and cover-slips are then treated separately with nitric acid. A 10 per cent. solution is usually sufficient, but occasionally the strong acid will be found necessary. Some workers prefer equal parts of alcohol and hydrochloric acid; still others the following mixture:

Bichromate of potassium,	2 parts;
Sulphuric acid,	3 "
Water,	25 "

A thorough washing in running water, followed by alcohol, completes the process. Alkalies are not so good for cleaning purposes, because they attack the glass.

Staining Dishes.—Watch-glasses are not satisfactory, on account of their instability. Concave dishes with flat bottoms are much better for ordinary use, and can be obtained of several patterns. They should be large enough to hold 25 c.c. of fluid. The *Syracuse solid watch-glasses* are very good dishes of this shape.

Stender dishes (Fig. 107) of various sizes will be found useful for many purposes.


BAUSCH-LOMB OPT. CO.
B.L.O.Co.
B.L.O.Co.
B.L.O.Co.

Sectional view.

FIG. 107.—Stender dishes.

Oblong rectangular Petri dishes are very convenient for staining preparations mounted on the slide.

Steinach's sieve-dish is valuable where a number of sections are to be stained in the same manner.

Large concave dishes holding 100 c.c. will be found the most convenient for holding frozen sections of fresh tissue, because a slide can be dipped into them and under the sections.

Metal Instruments.—Spatulas of different sizes are needed. They should be thin, smooth, and large enough, so that a section will not curl over the edge (Fig. 108).

The best instrument for transferring sections under all circumstances is a piece of platinum wire mounted in an ordinary screw needle-holder. It is pliable and can be bent to any shape, will not break like a glass needle when dropped, and is not affected by acids. Ladies' hat-pins form a cheap but serviceable substitute. They are readily bent to any desired shape by heating. Forceps, scissors, scalpels, and many other instruments required in microscopical work do not need any special mention.

FIG. 108.—Spatula.

Bottles.—For cover-slip work and for staining on the slide dropping-bottles will be found extremely convenient. The patent T. K. pattern of 50 c.c. capacity is probably the best form and size.

EXAMINATION OF FRESH MATERIAL.

Fresh tissues may be examined either in teased preparations or in sections.

Teased preparations are made by cutting out a very small bit of the tissue in question and dividing it as finely as possible, by means of two sharp, clean needles, on a slide in a drop or two of some indifferent fluid, such as the normal salt solution. Teased preparations are often made, for instance, of the heart-muscle when fatty degeneration is suspected. If the tissue is soft, the cells are easily obtained by simply scraping the cut surface with the edge of the knife.

Sections of fresh tissues can be made with a razor or with a double knife, but much the better way, at least for general diagnostic purposes, is to use frozen sections, which can be very quickly and perfectly made with the freezing microtome. The fresh sections are put into salt solution in a glass dish large enough to permit of a slide being dipped into it, so that a section can be floated and spread out evenly on its surface. The slide is then carefully raised, the excess of fluid wiped off, and a cover-slip put on.

If it is desired to stain the section, a few drops of Löffler's methylene-blue solution are poured over it after it is spread evenly on the slide. In a few seconds the coloring fluid is thoroughly washed off with salt solution, a cover-slip put on, and the section examined in the salt solution. If sections of fresh tissues are put directly into a staining fluid in the ordinary manner, they pucker up and do not stain evenly.

Fresh preparations are often treated with chemicals for various purposes. Of these chemicals, *acetic acid* is the most generally useful in pathological work. It shrinks the nuclei and renders their outlines more distinct. It swells connective tissue, making it more transparent, so that the elastic fibers which are unaffected stand out distinctly. It precipitates mucin and dissolves or renders invisible the albuminous granules so abundantly present in the protoplasm in the cloudy swelling of various organs in disease. Its main use as a reagent for fresh tissues is to demonstrate fat and to differentiate that substance from albuminous granules.

Acetic acid is ordinarily used in a 1 to 2 per cent. aqueous solution, a few drops of which are placed at one edge of the cover-slip, and then drawn beneath it by placing a piece of filter-paper on the opposite side. If in a hurry, however, stronger solutions, or even glacial acetic acid, may be used. Other reagents are of less importance, but are occasionally used.

Osmic acid is sometimes employed in a 1 per cent. aqueous solution to demonstrate fat, which it stains brown to black.

An alcoholic solution of *Sudan III* is being used more and more for the same purpose. It stains fat orange to red.

Hydrochloric acid in a 3 to 5 per cent. solution is used to demonstrate calcification. Phosphate of lime is simply dissolved, while from carbonate of lime bubbles of carbon-dioxid (CO_2) are set free.

Indifferent Fluids.—Fresh tissues are usually examined in normal salt solution, a $\frac{6}{10}$ per cent. solution of common salt in water. It has the advantage over water that tissues do not swell up so much in it, blood-globules are unaffected, and the finer structures are better preserved. A very few drops of Lugol's solution added to the stock-bottle of salt solution will be found useful in preventing the growth of mould.

Serous fluids, such as hydrocele fluid, are occasionally used. Artificial serum is made by adding 1 part of egg-albumin to 9 parts of normal salt solution.

Macerating fluids are little used in pathology. Occasionally, however, when tissues are tough, so that they cannot be readily teased apart, they are macerated in certain fluids which dissolve the substances that hold the different elements together. The reagents most commonly used are the following:

1. *Ranvier's one-third alcohol* is made by taking 1 part of 96 per cent. alcohol and 2 parts of water; twenty-four hours are usually enough.

2. Very dilute solutions of *chromic acid* are recommended —$\frac{1}{100}$ to $\frac{1}{300}$ of 1 per cent.

3. *33 per cent. Caustic Potash.*—Tissues are macerated in a few minutes to one hour: they must be examined in the same fluid, because the cells are destroyed if the solution is weakened.

Examination of Fluids.—Small fragments of tissue should be picked out with forceps. If much blood is adherent, wash the tissue well in salt solution. When the cellular elements are few in number they are obtained with a pipette, just as in urine-work, after allowing them to settle at the bottom of the glass. A centrifugal machine will be found of great service when the sediment is slight.

INJECTIONS.

INJECTIONS are not much used in pathology. The process is an art that requires much patience and considerable experience. The purpose of an injection is to render vessels and vessel-walls more visible than under ordinary circumstances. Transparent, deeply-colored fluid mixtures are used which will become hard in the vessels. Some injection-masses are employed cold, others warm. The warm injection-masses contain gelatin, and are much more troublesome to use, but give much the more perfect results. For coloring the mass carmine is the best material, because it is a permanent color.

The instruments required are cannulas of various sizes and a syringe, or, better still, a constant-pressure apparatus.

When a warm injection-mass is used the bottle containing the mass must be placed in a water-bath and kept at a temperature of about 45° C. The organ or animal to be injected must likewise be placed in a water-bath of the same temperature.

It is very important that in connecting the end of the tube carrying the injection-mass with the cannula inserted in the vessel no air-bubbles shall enter. When blood-vessels are to be injected it is advisable to wash them out first with normal salt solution.

Cold Injection-masses.—1. *Blue Coloring Mass.*—

Soluble Berlin blue,	1;
Distilled water,	20.

2. *Carmine Injection-mass* (Kollmann).—Dissolve 1 gram of carmine in 1 c.c. of strong ammonia plus a little water; dilute with 20 c.c. of glycerin. To this solution add 1 gram of common salt (NaCl) dissolved in 30 c.c. of glycerin. To the whole solution add an equal quantity of water.

Warm Injection-masses.—1. *Berlin Blue.*—Warm the solution of Berlin blue given above, and add it, with continual stirring, to an equal quantity of a warm, concentrated solution of gelatin prepared as follows: Allow clean sheets of the best French gelatin to swell up for one to two hours at room-temperature in double the quantity of water. Then dissolve them by warming gently over a water-bath. Filter the combined solution through flannel.

2. *Carmine-gelatin Mass.*—This is by all means the best injection-mass to use, because it is permanent, but it is very difficult to prepare.

Dissolve 2 to 2.5 grams of best carmine in about 15 c.c. of water, to which just enough ammonia is added, drop by drop, to effect the solution. Filter the fluid obtained, and add it, with continual stirring, to a filtered warm, concentrated solution of gelatin (prepared as above) over the water-bath. Then add acetic acid slowly until the color changes to a bright-red shade. The exact amount desired is when the solution loses its ammoniacal odor and has a peculiar sweetish aroma free from acid. Examined under the microscope, no granular precipitate of carmine should appear. If too much acetic acid has been added, so that the carmine is precipitated, the mass must be thrown away and a new lot prepared.

Organs which have been injected with a cold mass are placed directly in 80 per cent. alcohol. After a few hours they are to be cut up into pieces that are not too small. After a warm injection-mass the organ or animal is placed first in cold water to hasten the solidification of the gelatin, and then transferred to 80 per cent. alcohol. Masses already prepared for injecting cold or warm can be obtained from Gruebler.

FIXING REAGENTS.

The various reagents used for the preservation of fresh tissues possess the properties of penetrating, killing, fixing, hardening, and preserving in different degrees. Of these properties "fixing" is the most important, and to a certain extent implies or includes the others. The term "fixative" has been used more particularly, perhaps, for reagents which preserve faithfully the various changes of the nucleus in karyomitosis. In a broader sense, however, it refers to the faithful preservation of any tissue-element or pathological product, and of the chemical properties peculiar to that element or product. A good fixative is a reagent that penetrates and kills tissues quickly, preserves the tissue-elements, and particularly the nuclei, faithfully in the condition in which they are at the moment when the reagent acts on them, and hardens or so affects them that they will not be altered by the various after-steps of staining, clearing, and mounting. Most fixatives are mixtures of different reagents so combined that all the desirable properties may be present in as large a degree as possible.

The choice of the proper fixing reagent for a given tissue is often difficult, and must depend largely on the nature of the pathological lesions present or suspected, and on the purposes for which the tissue is preserved. For diagnosis, for general bacteriological study of tissues, and for many valuable and important chemical reactions alcohol is to be preferred to any other reagent. Although by no means an ideal fixative, it will be found exceedingly useful.

For finer histological study it is important to preserve valuable tissue in some more perfect fixative than alcohol. Zenker's and to some extent Orth's fluid will be found the most generally useful. Flemming's solution is especially to be recommended for the study of renal lesions with fatty degeneration. For general pathological work, aside from the nervous system, these four fixatives will be found the most valuable.

It is strongly advised that in all important cases pieces of tissue be hardened both in alcohol and in Zenker's fluid—in alcohol for bacteria and for chemical reactions; in the other for bacteria, nuclear figures, blood, and general histological study. For special investigations other fixatives are sometimes desirable.

Tissues fixed in alcohol or in a solution of formaldehyde may remain as long as desirable in those fluids. Tissues hardened in most of the other fixatives must be transferred, after thorough washing in water, to alcohol for preservation. It is usually recommended to pass the specimens through graded alcohols, either through 30, 60, 90, and 96 per cent., or through 50, 70, and 96 per cent., allowing them to remain from a few hours to a day in each strength. For most purposes it will be found sufficient to transfer the specimens directly from water to alcohol of 70 to 80 per cent., in which they may remain until it is desired to imbed them.

Alcohol extracts chrome salts from tissues hardened in solutions of them. As these salts are precipitated in the alcohol under the action of light, it is desirable, although by no means necessary, to keep all such specimens in the dark.

Alcohol.—The strength of alcohol ordinarily used in laboratories is 95–96 per cent. Absolute alcohol is much more expensive. Tissues hardened in either of these strengths shrink a great deal. The exposed surface becomes extremely hard, and the outer layers of the cells of tissues like a rabbit's kidney, for example, are as shrunken and flattened as though dried in the air. It is only inside of this hard casing, where the alcohol has penetrated more slowly and has been somewhat diluted by the fluid of the tissue, that the cells are better preserved. Moreover, this extreme hardening of the surface hinders the penetration of the alcohol into the deeper parts.

Tissue which is to be hardened in absolute or 95 per cent. alcohol should be cut into thin pieces, preferably not over ½ cm. thick. The volume of alcohol used for hardening should be fifteen to twenty times as great as the specimen, and should be changed after three or four hours. The tissue should be kept in the upper part of the alcohol by means of absorbent cotton, or the jar may be frequently inverted and the alcohol thus kept of even strength.

The advantages of strong alcohol, 95 per cent. and absolute, are that the tissue is more quickly fixed than with weaker strength, and that at the same time it is made quite hard—a quality more necessary formerly than now when tissues are so generally imbedded. Tissues hardened in strong alcohol should later be transferred to 80 per cent. alcohol for preservation or the staining properties will gradually become impaired.

For general purposes it will be found better to place tissues at first into 80 per cent. alcohol, which should be replaced in two to four hours by 95 per cent. alcohol. In this way less shrinkage is caused and the surface of the tissues is not made so hard.

Zenker's Fluid.—

Bichromate of potassium,	2.5	grams;
Sulphate of sodium,	1	"
Corrosive sublimate,	5	"
Glacial acetic acid,	5	c.c.;
Water,	ad 100	"

The solution is practically Müller's fluid saturated with corrosive sublimate, plus 5 per cent. of glacial acetic acid. It is advisable not to add the acetic acid to the stock solution, but only in the proper proportion to the part taken for hardening pieces of tissue, because the acid evaporates so readily.

Directions for Use.—1. Fix tissues in the solution twelve to twenty-four hours, rarely forty-eight hours, according to thickness.

2. Wash in running water twelve to twenty-four hours.

3. Preserve in 80 per cent. alcohol until used.

Tissues float at first in this solution, which will be found a most admirable general fixative. It penetrates quickly, so that pieces of tissue do not need to be so thin as with most other fixatives, but it is advisable not to let them exceed 4 to 6 mm. in thickness. Nuclear figures, red blood-globules, and protoplasm are all perfectly preserved. The greatest drawback to the fluid is the precipitation of mercury, which takes place to a varying degree in the tissues. This precipitation can be removed by means of iodin, which forms a colorless, soluble compound. For this purpose it has usually been advised to add iodin to the alcohol in which the tissues are preserved. Prolonged treatment, however, is required, and iodin exerts an injurious effect on the staining properties of the cells, so that in general it will be found best to imbed the tissues and cut sections without removing the precipitate, and to treat the sections, just before staining, with Lugol's solution or a 1 per cent. alcoholic solution of iodin for ten to twenty minutes, followed by alcohol to remove the iodin. In old Zenker preparations the areas in the tissues occupied by the mercuric precipitate often stain with alum hematoxylin, although the precipitate itself has been removed.

Zenker preparations stain slowly but beautifully in alum-hematoxylin. Excellent results can also be obtained with eosin, followed by Unna's alkaline methylene-blue solution. Fuchsin and safranin stains are sometimes useful.

Corrosive Sublimate.—Use a saturated solution (made by heat) in normal salt solution. The addition of 5 per cent. of glacial acetic acid is usually advisable. 1. Harden thin pieces of tissue (2 to 5 mm.) for six to twenty-four hours; 2. Wash in running water twenty-four hours; 3. Preserve in 80 per cent. alcohol.

Tissues hardened in corrosive stain quickly and brilliantly in nearly all staining solutions. It is the only fixative after which the Heidenhain-Biondi triple stain gives good results.

Picric Acid.—Use a saturated aqueous solution. Fix tissues 4 to 6 mm. thick for twelve to twenty-four hours. Do not wash out in water, but extract the picric acid by repeated changes of 80 per cent. alcohol.

Orth's Fluid.—Recently Orth has highly recommended as a general fixative a solution consisting of the well-known Müller's fluid plus 4 per cent. of formaldehyde:

Bichromate of potassium,	2 to 2.5;
Sulphate of sodium,	1;
Water,	100;
Formaldehyde (40 per cent. solution),	10.

The formaldehyde should be added only at the time of using, for in two days the solution becomes darker, and by the fourth day a crystalline deposit begins to take place. As fixation is ordinarily complete in three to four days, this deposit does not matter. The tissue should not be over 1 cm. in thickness. Small pieces $\frac{1}{3}$ to $\frac{1}{2}$ cm. in thickness can be readily hardened in the incubator in three hours. The specimens should be washed thoroughly in running water six to twenty-four hours before placing in 80 per cent. alcohol.

The method is particularly recommended for mitosis, red blood-globules, bone, and colloid material (in cystomata, etc.), as it gives a very good consistency to the tissues, but the histological detail is not so good as after Zenker's fluid.

Flemming's Solution.—

Osmic acid, 2 per cent. aqueous solution,	4;
Chromic acid, 1 per cent. aqueous solution,	15;
Glacial acetic acid,	1.

1. Fix in the solution one to three days. 2. Wash in running water six to twenty-four hours. 3. Alcohol, 80 per cent.

It is best to keep the osmic acid in a 2 per cent. solution, and the chromic acid in a 1 per cent. solution. The mixture can then be quickly made up fresh at the time it is needed. The best stains after hardening in Flemming are Babes' safranin, aniline-gentian-violet, and carbol-fuchsin.

Pieces of tissue for hardening in Flemming's solution should not be over 2 mm. in thickness, because it has very slight penetrating properties.

Hermann's Solution.—

Osmic acid, 2 per cent. aqueous solution,	4;
Platinic chlorid, 1 per cent. aqueous solution,	15;
Glacial acetic acid,	1.

This modification of Flemming's solution is, perhaps an even better fixative than the model on which it is based, but is more expensive. It should be employed in the same manner.

Pianese's Solution.—

Chlorid of platinum and sodium, 1 per cent. aqueous solution (platinic),	15 c.c.
Chromic acid, ¼ per cent. aqueous solution,	5 "
Osmic acid, 2 per cent. aqueous solution,	5 "
Formic acid, C. P.	1 drop.

Fix small pieces of tissue, not over 2 mm. thick, in the solution for thirty-six hours. Wash in running water for twelve hours, then 80 per cent. alcohol. Stain paraffin sections by Pianese's special methods (see p. 275).

This fixative and the special staining methods are particularly recommended for the study of karyomitosis and of the so-called cancer bodies.

Rabl's Chromo-formic Acid Solution.—

Chromic acid, 0.33 per cent. aqueous solution,	200;
Formic acid,	4 to 5 drops,

to be added just before the solution is used.

Directions for Use.—1. Harden in the fixing solution twelve to twenty-four hours; 2. Wash in running water twelve to twenty-four hours; 3. Dehydrate in 80 per cent. alcohol.

Rabl used after this fixative a very faint stain with hematoxylin, followed by safranin.

Formaldehyde.—The gas formaldehyde (HCOH) is soluble in water to the extent of 40 per cent. Solutions of this strength are manufactured by different commercial houses under the names of formaline, formol, and formalose. The best strength of formaldehyde to use for fixing tissues is a 4 per cent. solution; that is, 10 parts of the aqueous 40 per cent. solution, no matter what name is given to it, to 90 parts of water—or, better still, perhaps, of normal salt solution.

This new fixing reagent penetrates very quickly. Its hardening action is not understood. It does not precipitate albuminous bodies, but makes them quite firm. It also hardens nerve-sheaths, acting toward them and red globules like the chrome salts. Formaldehyde is very useful for preserving gross specimens, because it gives them a rather tough, elastic consistency, and preserves the normal colors better than other hardening fluids, and also the transparency of many parts, such as the cornea. In histological work it has been found most useful, so far, for the preservation of nervous tissue.

Sections of ordinary tissues not over 1 cm. thick are hardened in twenty-four hours. They may then be transferred to alcohol, or may remain indefinitely in the formaldehyde. Large pieces require more time. Formaldehyde is much used for hardening tissues quickly, so that frozen sections can be cut and permanent preparations stained and mounted in the course of a few hours. Thin pieces are sufficiently hardened in one to three hours. They may be frozen directly in the formaldehyde solution or in water. Almost any stain is applicable after the sections have been washed in water and treated for a short time with alcohol.

Although formaldehyde is an excellent fixative from the point of view of killing quickly, it does not seem to preserve tissue-elements so that they will not later undergo changes when treated with various reagents. For this reason it is best combined with some more purely hardening reagent, as, for example, bichromate of potassium in Orth's solution. Tissues such as muscle or the contents of a multilocular cystoma which have been made very hard by the fixing reagent used, can be softened, even when mounted in celloidin, so that they will cut perfectly, by placing in a 4 per cent. solution of formaldehyde for twenty-four hours.

Boiling.—Boiling precipitates the soluble albumin in tissues as a granular material which can be readily recognized. The method is used particularly for the demonstration of albumin in renal diseases and in edema of the lungs. By means of boiling the quickest permanent mounts of tissues can be obtained. The method is not advocated, on account of the shrinkage caused by the heat, but will sometimes be found useful.

Small pieces of tissue not over 1.5 cm. in diameter should be dropped into the boiling water for one half to two minutes; cool quickly in cold water and make frozen sections, or put into 80 per cent. alcohol. Any stain can be used; methylene-blue will be found excellent.

Müller's Fluid.—

Bichromate of potassium,	2 to 2.5 grams;
Sulphate of sodium,	1 "
Water,	100 c.c.

Harden tissues six to eight weeks. Change the fluid daily during the first week; once a week thereafter. Ordinary tissues are then washed in running water over night before being placed in alcohol. Nervous tissue is transferred directly from the fluid to the alcohol.

This famous hardening solution seems destined before long to give way entirely to better fixatives. It hardens tissues slowly, evenly, and with little or no shrinkage, but it is a poor nuclear fixative, and does not encourage any great variety of stains. For ordinary tissues it will undoubtedly be replaced by Zenker's or Orth's fluid, both of which fix very quickly, besides having all its good qualities. For nervous tissues formaldehyde followed by other solutions of the chrome salts is a great deal quicker and better.

Marchi's Fluid.—

Müller's fluid,	2 parts;
Osmic acid, 1 per cent. aqueous solution,	1 part.

Place small pieces of tissue in the mixture for five to eight days, wash thoroughly in running water, and harden in alcohol. For its application to degenerated nerve-fibers see page 332.

Erlicki's Fluid.—

Bichromate of potassium,	2.5 grams;
Sulphate of copper,	1. "
Water,	100 c.c.

Hardening is quicker than with Müller's fluid, requiring eight to ten days, otherwise the treatment is the same.

DECALCIFICATION.

TISSUES which are to be decalcified should be sawn with a fine hair-saw into thin slices, so that they will decalcify quickly. It is usually desirable to saw the tissue into pieces of proper size for imbedding in celloidin. Very dense bone ought not to be over 2 or 3 mm. thick; softer tissues do not need to be thinner than 4 to 6 mm. In cutting sections after decalcifying and imbedding it is necessary to throw away the first half-dozen sections or so, because the tissue is so lacerated to a slight depth by the movement of small fragments of bone in the process of sawing as to be useless for microscopic purposes. The extent of the decalcification can be tested at any time by thrusting a needle into the tissue.

The following steps in the decalcification of tissues must be carefully borne in mind:

1. The tissues must first be thoroughly hardened. The three most useful reagents for this purpose are alcohol and Zenker's and Orth's fluids. After the two latter reagents the tissues must have been washed thoroughly in water and placed in alcohol for at least twenty-four hours. They will then be ready for decalcification.

2. The decalcifying fluid must be used in large amounts, and if necessary be frequently changed.

3. After decalcification the tissues must be thoroughly washed in running water for twenty-four hours to get rid of every trace of the acid.

4. The tissues finally must be hardened again in alcohol.

Of the various agents used for decalcifying bone, nitric, hydrochloric, chromic, picric, trichloracetic acids, etc., the most important is nitric acid. It acts quickly, without swelling the tissues or attacking injuriously the tissue-elements, and does not interfere to any marked degree with any subsequent staining process. Red blood-globules will be found uninjured in tissues hardened in Zenker's fluid even after remaining four days in 5 per cent. nitric acid. This acid is used in dilute solution alone or in combination with phloroglucin.

Directions for Using Nitric Acid.—1. Decalcify in large quantities of a 5 per cent. aqueous solution of nitric acid, changing the solution every day for one to four days. 2. Wash twenty-four hours in running water to remove every trace of acid. 3. Harden in 80 per cent., and then 95 per cent., alcohol. Imbed in celloidin.

Phloroglucin and Nitric Acid.—Phloroglucin is not a decalcifying agent, but is added to nitric acid to protect the tissues while allowing a stronger solution of the acid to be used than would otherwise be possible. The solution is prepared by dissolving 1 gram of phloroglucin in 10 c.c. of nitric acid. Solution takes place quickly, with generation of considerable heat. The fluid is reddish brown at first, but becomes light yellow in the course of twenty-four hours. Dilute with 100 c.c. of a 10 per cent. solution of nitric acid. This gives nearly a 20 per cent. solution of nitric acid. The process of decalcification in this fluid is extremely rapid; a few hours only, as a rule, are required. It is not advisable to dilute the solution by the simple addition of water, but by the use of less acid, because the phloroglucin must be present to the amount of 1 per cent. or it will not protect the tissues so well.

The following slower-acting solution may be found useful:

Phloroglucin,	1;
Nitric acid,	5;
Alcohol,	70;
Water,	30.

A rather deep single stain with alum-hematoxylin (either aqueous solution or Delafield's) will usually be found to give the best results with tissues decalcified with nitric acid. It is very important to leave the sections after staining in a large dish of water over-night, otherwise the stain will not be so sharp and clear.

Picric Acid.—A saturated aqueous solution containing an excess of crystals is sometimes used for decalcifying. It has no injurious action on tissues, but is extremely slow, frequently requiring months. Fresh tissues may be placed directly in the solution, which hardens and decalcifies at the same time. Instead of being washed out in water, in which they would macerate, the pieces of tissue are placed directly in 70 or 80 per cent. alcohol to remove the acid.

Hydrochloric Acid.—*v. Ebner's Decalcifying Solution:*

Hydrochloric acid,	2.5;
Alcohol,	500.0;
Water,	100.0;
Chloride of sodium,	2.5.

The action of this solution is slow, and but slightly injurious.

Trichloracetic Acid.—A 5 per cent. solution of this acid has lately been recommended for the decalcification of bone and teeth. It acts more slowly than nitric acid, and seems to possess no advantages over it. Tissues must be washed out in running water, as after nitric acid.

IMBEDDING PROCESSES.

Sections of hardened tissues can be cut with a razor by hand, or with a microtome knife after fastening the specimen in the microtome clamp either directly or between pieces of amyloid liver. Fair sections of firm tissues can often be obtained in this way. Thinner sections can be got by means of the freezing microtome, but these methods are all open to the objection that unless the tissue is very cohesive portions of it are likely to fall out of the sections.

The best results would, therefore, naturally be expected from some imbedding process, employing a substance to infiltrate the tissues thoroughly and to hold the different parts in proper relative position even in the thinnest sections.

The two substances in common use for this purpose are celloidin and paraffin. Each has its advantages and disadvantages. Neither can be employed in pathological histology to the exclusion of the other. Paraffin affords the thinner sections, but they must be small if the best results are desired, and cannot be properly handled except when fastened to the slide. Hard tissues like muscle, and tissues of varying consistency like skin, are cut with considerable difficulty by the paraffin method. Staining is rather simpler than after imbedding in celloidin.

On the other hand, tissues of almost any consistency or size can be cut by the celloidin method, which is also capable of furnishing very thin sections.

Both methods of imbedding should be learned and used. Celloidin sections are especially good for general work, for studying the extent and relations of pathological processes, and for much of the finer histological work. Paraffin sections are better for the finest details of processes—for special work on special tissues.

Celloidin.—Schering's celloidin is the best preparation of gun-cotton (pyroxylin) to use. It is sold now in a convenient granular form in small bottles. It keeps well, dissolves somewhat slowly, and gives a fairly transparent imbedding mass which is firm and tough, so that very thin sections can be cut. Other forms of gun-cotton are not so reliable; they often contain impurities and do not yield so firm an imbedding mass.

Imbedding in Celloidin.—The process consists in soaking the tissues for twenty-four hours to a number of days in two different solutions of celloidin. The two solutions are spoken of as thin and thick celloidin. To make thick celloidin 30 grams of the dry celloidin are dissolved in 500 c.c. of a mixture of equal parts of ether and absolute alcohol. This gives a 6 per cent. solution. Diluted with an equal amount of the ether-and-alcohol mixture, it forms thin celloidin.

The steps of the imbedding process are as follows: Pieces of tissue which have been properly fixed and finally preserved in 80 per cent. alcohol are first to be cut up with intelligence. They should rarely be over 4 to 8 mm. thick; for most purposes 2 mm. will be found sufficient. Pieces of this thickness will furnish several hundred sections, will imbed more quickly than larger masses, and will be more rigid when mounted on a block. They should never be broader or longer than is necessary to show the whole of the process under study. Very thin celloidin sections cannot usually be obtained with tissues over 1½ to 2 cm. square, and smaller dimensions are preferable. Beginners usually imbed larger pieces than are necessary.

The trimmed pieces of tissue are first hardened and dehydrated for twenty-four hours in 95 per cent. alcohol, followed by twenty-four hours in absolute alcohol; then soaked in equal parts of absolute alcohol and ether for the same length of time to prepare them for the thin celloidin. In the latter they remain at least twenty-four hours, preferably for a number of days if at all thick, for in this solution occurs most of the infiltration with celloidin. Finally, the pieces are soaked twenty-four hours or more in the thick celloidin. They are then mounted on blocks of vulcanized fiber, exposed to the air for two or three minutes till the surface hardens a little, and placed in 80 per cent. alcohol for six to twenty-four hours to allow the celloidin to harden.

Briefly summed up, the steps of imbedding in celloidin are as follows:

1. 95 per cent., followed by absolute alcohol, twenty-four hours each.
2. Ether and absolute alcohol, *āā.* twenty-four hours.
3. Thin celloidin, twenty-four hours to one or more weeks.
4. Thick celloidin, twenty-four hours to one or more weeks.
5. Mount on blocks of vulcanized fiber: dry a minute or two in the air.
6. Harden celloidin in 80 per cent. alcohol, six to twenty-four hours.

The second step may be omitted if time is pressing.

Instead of mounting directly from the thick celloidin, it is often advisable to allow the celloidin to evaporate until a firm mass is obtained. This is particularly true when very loose tissues are to be imbedded.

The simplest method is to place the pieces of tissue, which have been soaking in thick celloidin, in proper position in a glass dish and pour thick celloidin over them. The dish is then covered, but not too tightly, and the ether is allowed to evaporate for one or more days until the proper consistency of celloidin is reached, so that it can be cut out in blocks enclosing the specimens. If the ether evaporates too rapidly, place a large dish or a bell-jar over the covered dish. Mount the blocks, after they have been cut out and trimmed, by dipping the bases in thick celloidin and then pressing them on to blocks of vulcanized fiber. In two or three minutes they can be placed in 80 per cent. alcohol. After the celloidin mounts have been in 80 per cent. alcohol for six to twenty-four hours the celloidin is of the proper consistency for cutting. It is best to take a sharp knife or an old razor and trim the top of the celloidin down to where the first good section of the specimen can be cut; this will save considerable wear on the microtome knife.

Stepanow[1] has recently recommended the following method of embedding in celloidin for three reasons: it is quicker, the infiltration is more perfect, and thinner sections are possible.

1. Dehydrate in alcohol, 95 per cent. or absolute.
2. Oil of cloves, three to six hours or longer.
3. Place in the following solution for three to six hours or longer:

Celloidin (dry and in fine granules),	1.5 g.;
Oil of cloves,	5.0 c.c.;
Ether,	20.0 c.c.;
Absolute alcohol,	1.0 c.c.

4. Pour the block of tissue and enough of the solution to cover it into a filter of very fine paper, and allow the solution to thicken, preferably in a warm place. As the solution thickens it becomes clear.
5. Cut out the block of tissue, and mount in the usual way.
6. Place in 80 per cent. alcohol (or in chloroform for one to two hours, followed by 80 per cent. alcohol).

In cutting, the microtome knife should be fastened very obliquely, so that as much of the edge of the knife as possible shall be used in making each section. The surface of the knife should be kept well wet with 80 per cent. alcohol, preferably from an overhanging drop-bottle.

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[1] *Zeitschrift f. wissensch. Mikroscopie*, 1900, xvii., 185.

If the sections curl, as often happens when they are thin, they are best flattened by unrolling them on to the surface of the knife with a camel's-hair brush just before the last edge of celloidin is cut through, as this serves to keep them fixed in place during the process. This method can be used when the simple transferring of sections from alcohol to water is not sufficient to uncurl them.

Celloidin sections can be stained by nearly all methods, without the necessity of removing the celloidin. When necessary, however, the celloidin is readily removed by placing the sections from absolute alcohol in oil of cloves or in the alcohol-and-ether mixture for five or ten minutes, and then passing them back through absolute into ordinary alcohol.

To Attach Celloidin Sections to the Slide.—A celloidin section can be fairly well attached to a slide by transferring it from water to a slide freshly washed in alcohol and dried with a cloth. The section is then to be firmly blotted with filter-paper so as to apply it closely to the slide and to remove all wrinkles. It should not be allowed to dry. A section treated in this way will ordinarily stand considerable manipulation without becoming loose.

Celloidin sections can be more securely attached by transferring them from 95 per cent. alcohol to clean slides and pouring over them ether-vapor from a bottle half full of ether. With a little practice sections can be fastened in a few seconds. Follow slowly along the edge of the celloidin, and the frills in it will soften down. Then wash the specimen with 80 per cent. alcohol to harden the celloidin.

Imbedding in Paraffin.—Paraffin imbedding is particularly useful when very thin sections are desired. To obtain the best results the pieces of tissue should be small, soft, and of uniform consistency. In pathological work it is much better to cut the sections and to stain them after they are fastened to the slide than to stain in the mass beforehand, because then a variety of stains may be used. A complete or perfect series is not so important as in embryology, but with a little care can be obtained.

The first step in the preparation of hardened tissues for the paraffin bath is to cut them into small, thin square or rectangular pieces, not over 1 cm. square, perhaps, for the best results, and not over 2 to 3 mm. thick. It should be stated, however, that with proper skill, a heavy, sharp knife, and a rigid microtome very thin paraffin sections can be obtained with tissues measuring 4 × 3 cm. The pieces of tissue are then thoroughly dehydrated by soaking first in 95 per cent. and then in absolute alcohol. From alcohol they are put in some substance, such as chloroform or oil of cedar, which has the property of mixing with alcohol and of dissolving paraffin. From the chloroform they are transferred to a saturated solution of paraffin in chloroform, and then passed through two separate baths of the melted paraffin to get rid of every trace of the chloroform. If oil of cedar is used, the specimens are transferred directly from it into the melted paraffin.

One advantage of the chloroform method is that the duration in the hot paraffin, the objectionable feature of the paraffin method, is shortened, because the tissues are already somewhat infiltrated with paraffin. Another advantage is that the paraffin bath purifies itself, because the chloroform rapidly evaporates. When oil of cedar is used the paraffin must be renewed frequently.

The methods of imbedding in paraffin are briefly stated as follows:

Method No. 1.

1. 95 per cent. alcohol,	6–24	hours.
2. Absolute alcohol,	6–24	"
3. Chloroform,	6–24	"
4. Chloroform saturated with paraffin,	6–24	"
5. Paraffin bath, two changes,	2–4	"

6. Imbed and cool quickly in cold water.

Method No. 2.

1. 95 per cent. alcohol,	6–24 hours.
2. Absolute alcohol,	6–24 "
3. Oil of cedar, two changes,	6–24 "
4. Paraffin, three changes, till no odor of oil of cedar.	2– 8 "
5. Imbed and cool quickly in cold water.	

In the second method other substances than oil of cedar can be used, such as xylol, equal parts of oil of cloves and turpentine, or oil of cloves and xylol.

For imbedding paraffin specimens metallic boxes can be used, or forms made round or square from strips of sheet lead or tin. Many prefer paper boxes, which can be made easily of any size desired from stiff writing-paper.

Melted paraffin is poured into the paper box to the depth of about 1 cm. The pieces of tissue are then placed in the box with that side down from which sections are preferred. When all of the pieces are arranged in order with about half a centimeter or more between them, the box is placed on the surface of a large dish of cold water, on which it floats, so that the paraffin may cool quickly without crystallizing. After the paraffin has hardened the paper is removed and the paraffin is divided up according to the pieces in it. One of the blocks is then fastened to the object-holder by heating the latter in a flame until it will just melt the paraffin when the block is held in proper position against it. The holder is then quickly cooled in cold water.

The upper surface of the paraffin should now be shaved down to the specimen. The four sides are to be carefully trimmed; the upper and lower surfaces should be parallel and not cut too close to the specimen, otherwise the sections will not adhere to each other; the lateral surfaces should, as a rule, be cut close to the tissue, especially if very thin sections are desired, because if a rim of paraffin is left it is likely to cause wrinkling of the sections. The holder is finally carefully adjusted in the paraffin microtome.

To get good sections which will adhere to each other and form a ribbon the temperature of the room must be regulated to suit the degree of hardness of the paraffin used. An open window will often make all the difference needed to obtain good results. The harder the paraffin the warmer the room must be. The temperature can be raised by burning a Bunsen flame near the microtome or lowered by the presence of a lump of ice. It will often be found advantageous to dip the holder and paraffin block into ice water just before cutting sections.

The ribbons of sections as cut, usually a slideful, are laid on the surface of a large dish of warm water at about 44° C., and if necessary gently stretched so as to remove all wrinkles. Paint the surface of a slide with a thin layer of Mayer's glycerin-albumin mixture, wipe off all excess with a towel so that only a faint layer is left, dip the slide under the sections, arrange them in order, lift the slide, and drain off the water. The slide is then placed in a slanting position until dry, when it is put in the incubator for two to twelve hours at a temperature of 54° to 60° C. This process attaches the sections firmly to the slide.

To get rid of the paraffin in the sections they are treated with two or three changes of xylol, and then with absolute followed by 95 per cent. alcohol.

If for any reason the celloidin-and-oil-of-cloves mixture is used for attaching the sections to the slide, the paraffin is removed by means of xylol, followed by origanum or bergamot oil, and finally by 95 per cent. alcohol, because absolute alcohol will dissolve the celloidin.

Serial Sections by the Celloidin Method.—1. For Tissues in General.—With a little care perfect serial sections can be made by the following method, and each slide of sections can be stained in whatever way seems best. The specimen is imbedded, mounted on vulcanized fiber, and hardened in 80 per cent. alcohol in the usual way. In cutting moisten the microtome knife with 95 per cent. alcohol. As the sections are cut they are drawn up on the surface of the knife and arranged in regular order by means of a camel's-hair brush until a slideful is ready. They are then drawn on a clean and numbered slide held against the back of the knife. After being carefully arranged the sections are fastened to the slide by means of ether-vapor (see p. 258) poured over them from a half-full bottle. Care must be taken that every edge of the celloidin is fully softened down. The slides are then placed in a jar of 80 per cent. alcohol to be stained at leisure.

2. Another method, often convenient where the stain is of little importance, is as follows: The tissue is stained, in bulk, in alum-cochineal or some other staining fluid that will penetrate, and then imbedded in celloidin in the usual way. After being mounted on vulcanized fiber the specimen is hardened in chloroform instead of in 80 per cent. alcohol. From the chloroform the specimen is transferred to oil of thyme. After it is thoroughly penetrated by the latter it is ready to be cut. The knife is to be moistened with oil of thyme. The sections as cut are arranged on the knife, and then transferred to slides placed against the back of the knife. The slides covered with sections can be placed under a bell-jar as fast as they are ready until all are cut, because the oil of thyme evaporates slowly. Balsam and cover-slips can be added after the cutting is finished.

3. Darkschewitsch has recently proposed a comparatively simple method for preparing a series of celloidin sections. A glass cylinder without a neck, of about the diameter of the specimen to be cut, is filled with alcohol. Then a series of circles of filter-paper are cut of a size just to fit the bottle, numbered in order, and wet with alcohol. Each section is removed from the microtome knife by pressing one of the paper circles upon it and drawing it off. The paper is then inverted so that the section is uppermost, and deposited in proper order in the bottle, where the series forms a column, each section resting upon a numbered paper. The sections can be kept indefinitely. When ready to stain the alcohol is poured off, the sections washed with water if necessary, and then the staining solution poured into the bottle. Other reagents are used in the same manner, or sections can be treated with the reagents in flat plates, as they do not readily slip off the papers.

4. Weigert's method for a series of celloidin sections was designed especially for the nervous system and is rather complicated. The process depends on transferring the sections as cut to narrow strips of tissue-paper. To do this each section as cut is arranged in proper position close to the edge of the knife. Then a strip of tissue-paper twice as wide as the section is gently placed upon it, and the sections withdrawn from the knife. The success of the process depends on having but little alcohol on the knife, otherwise the specimen will not stick. Each specimen is placed on the paper to the right of the last one. The strips of paper when full are kept moist by being placed with the specimens uppermost on a moist surface composed of a layer of blotting-paper wet with alcohol, covered with a sheet of tissue-paper, and lying in a shallow dish.

When all the sections have been cut, each strip of them is taken in turn and coated on both sides with a thin film of celloidin in the following way: A strip of sections with the specimens below is first pressed gently down upon the surface of a slide covered with a thin layer of celloidin. This fastens the sections and the paper can be removed. Then a thin coat of celloidin is poured over the sections and the slide is placed on its edge to drain. When the surface of the celloidin is dry, the strips can be marked by a fine brush dipped in methylene-blue. As soon as the slides are placed in the staining solution the celloidin peels off, taking the specimens with it. Later, the strips of specimens can be divided as desired. On account of their thickness they should be cleared, after dehydrating in 95 per cent. alcohol, in a mixture of xylol 3, carbolic-acid crystals 1.

To obtain **serial sections by the paraffin method** it is only necessary to avoid losing any of the sections from the ribbon as ordinarily cut. Perhaps the easiest and safest way is to cut long ribbons, a yard or more in length, and to place them on sheets of paper in proper order. They can then readily be divided by means of needles into short series of any desired number of sections, and fastened to numbered slides by means of albumin fixative.

STAINING SOLUTIONS.

Hematoxylin and Hematein Stains.—The active coloring agent in most hematoxylin stains is hematein, which is gradually formed in the ordinary solutions from the hematoxylin by oxidation, a process occupying a number of days or weeks and spoken of as "ripening." The selective staining power of alum-hematoxylin solutions is due to the combination of this hematein with alumina. The resulting blue-colored solution is precipitated in the tissues (chiefly in the nuclei) by certain organic and inorganic salts there present, as, for instance, phosphates.

Mayer and Unna have shown that it is possible to oxidize and to ripen in an instant a solution of alum and hematoxylin by adding to it a little peroxid of hydrogen neutralized by a crystal of soda.

By employing hematein or its ammonium salt, instead of hematoxylin, Mayer has been able to obtain immediately ripened solutions which compare fairly favorably with old and well-known solutions prepared from hematoxylin by the slow process of ripening. They do not stain any better, however, and it is doubtful if, for the present at least, they become generally accepted.

Most solutions of alum and hematoxylin are not stable. A continuous chemical change is the formation from hematoxylin, by oxidation, of hematein, which, uniting with the alum, gives a bluish or purplish solution. The degree of blueness depends largely on the freshness of the alum. As the solution becomes older free sulphuric acid is gradually formed from the alum, causing the solution to lose its bluish or purplish tint and to become reddish. A third chemical change is the continuous formation of a precipitate due to the further oxidation of the hematein, in consequence of which it is always necessary to filter alum-hematoxylin solutions just before they are used.

More alum than is needed to combine chemically with the hematoxylin is always added to the solution, for the reason that it acts as a differential decolorizer, limiting the stain largely to the nuclei of the cells. As alum-hematoxylin solutions become older they stain more quickly, but also more diffusely. This diffuseness of staining can be counteracted by adding enough alum-water to make the stain precise again. A good alum-hematoxylin solution ought not to stain the celloidin in which the section is imbedded. If the celloidin stains more or less deeply, it shows that the solution requires more alum.

Aqueous Alum-hematoxylin Solution.—

Hematoxylin crystals,	1;
Saturated aqueous solution of ammonia alum,	100;
Water,	300;
Thymol,	a crystal.

The hematoxylin crystals are dissolved in a little water by the aid of heat. The combined solution is exposed to the light in a bottle lightly stoppered with a plug of cotton. The solution will be ripened sufficiently for use in about ten days, after which time it should be kept in a tightly stoppered bottle. The solution is very easily prepared, gives beautiful results, and will keep at its best for two to three months. The proportions of alum and of hematoxylin are the same as in Delafield's solution. For Zenker preparations, which stain very slowly, it will be found more convenient to omit the 300 c.c. of water in the above formula.

Delafield's Hematoxylin.—

Hematoxylin crystals,	4 grams;
Alcohol, 95 per cent.,	25 c.c.;
Saturated aqueous solution of ammonia alum,	400 "

Add the hematoxylin dissolved in the alcohol to the alum solution, and expose the mixture in an unstoppered bottle to the light and air for three to four days.

Filter, and add—

Glycerin,	100 c.c.;
Alcohol, 95 per cent.,	100 "

Allow the solution to stand in the light until the color is sufficiently dark, then filter and keep in a tightly-stoppered bottle. The solution keeps well and is extremely powerful. So long as it is good the solution has a purplish tinge.

It would seem advisable, both in this solution and in Ehrlich's, to combine the alum, hematoxylin, and the water, and to ripen the solution for two or three weeks before adding the other ingredients which have a tendency to prevent oxidation. A fully-ripened solution would then be obtained more quickly and surely.

Ehrlich's Acid Hematoxylin.—

Hematoxylin crystals,		2 grams;
Absolute alcohol,		60 c.c.;
Glycerin,	60 c.c.	saturated with ammonia alum.
Water,	60 "	
Glacial acetic acid,	3 "	

The solution is to be exposed to the light for a long time until it acquires a deep-red color. If it then be kept carefully stoppered, its staining powers will remain constant for years. The acetic acid is added to prevent the formation of insoluble compounds of hematein and as a decolorizer to limit the stain to nuclei.

Mayer's Hemalum.—

Hematein, or its ammonia salt,	1 gram;
90 per cent. alcohol,	50 c.c.;
Alum,	50 grams;
Water,	1000 c.c.;
Thymol,	a crystal.

Dissolve the hematein or its ammonia salt in the alcohol by the aid of heat, and add it to the alum dissolved in the water. The solution can be diluted with 20 parts of water or of weak alum solution.

Mayer's acid hemalum is prepared by adding 2 per cent. of glacial acetic acid to the above solution. The acid stain is more precise than the alkaline.

Mayer's Glycerin-alum-hematein Solution.—According to Mayer's latest investigations, glycerin is the only reliable preservative of hematein solutions. Unfortunately, it slows the staining power to a considerable extent and makes the stain less precise. He recommends the following solution for its keeping properties:

Hematein, (dissolve by rubbing up in a few drops of glycerin);	0.4 grams
Alum,	5 grams;
Glycerin,	30 c.c.;
Water,	70 "

Mayer's Muchematein.—

Hematein,	0.2 grams;
Chlorid of aluminum,	0.1 "
Glycerin,	40 c.c.;
Water,	60 "

Rub up the hematein with a few drops of glycerin, add the chlorid of aluminum, and dissolve the mixture in the glycerin and water.

Weigert's Alcohol Hematoxylin.—

Hematoxylin crystals,	10 grams;
Alcohol (absolute or 95 per cent.),	90 c.c.

The solution ripens in a week or two to a brown color, and keeps perfectly for a long time. It is used only in the Weigert stain for myelin sheaths, for which purpose it is diluted at the time of using with water and combined with carbonate of lithium (see page 324).

Phosphomolybdic Acid Hematoxylin (*Mallory*).—

Hematoxylin crystals,	1.75 grams;
½ per cent. aqueous solution of phosphomolybdic acid,	200 c.c.

The hematoxylin will dissolve almost immediately if powdered, or it may be dissolved in water by the aid of heat. The solution must be exposed to the light in a bottle plugged with cotton for five to six weeks before it is fully ripened. It will keep for several months, and can be used over and over. It is employed for staining the nervous system and connective tissue (see page 305).

Phosphotungstic Acid Hematoxylin (*Mallory*).[1]—When this formula was first published the phosphotungstic acid manufactured by Merck was not pure. It contained a trace of phosphomolybdic acid, and also some oxidizing agent which ripened hematoxylin at once. The staining solution made up according to the directions then given, but with the pure phosphotungstic acid now manufactured, gives negative results because the hematoxylin will not ripen spontaneously even after standing for months. The solution can be ripened, however, at once by the addition of peroxid of hydrogen.

Hematoxylin,	0.1	gram ;
Water,	80.	c.c. ;
10 per cent. aqueous solution of phosphotungstic acid (Merck),	20.	"
Peroxid of hydrogen (U. S. P.),	0.2	"

Dissolve the hematoxylin in a little water by the aid of heat, and add it after it is cool to the rest of the solution. The mixture is ready to use at once, keeps well, and requires no antiseptic.

It is used mainly for staining neuroglia-fibers, but gives interesting results with elastic fibers, striated muscle-fibers, fibrin, and cartilage.

Carmine Stains.—The active staining principle in carmine solutions is carminic acid. In cochineal carminic acid is combined with an alkaline base. Carmine itself is a commercial compound containing carminic acid combined with aluminum and calcium. Carminic acid itself does not stain, but it forms compounds with certain metals, mainly with the aluminum contained in alum, which have selective staining properties.

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[1] Mallory: *The Journal of Experimental Medicine*, 1900, v., 19.

All of the alkaline and acid solutions made with carmine owe their staining properties to carminic acid combined with the aluminum, and perhaps also to the calcium contained in the carmine.

Alum Carmine.—

Carmine,	2 grams;
Alum,	5 "
Water,	100 c.c.

Boil twenty minutes, adding enough water to make up for that lost by evaporation. When cool, filter and add a crystal of thymol to prevent the growth of mould.

Alum Cochineal.—

Powdered cochineal,	6 grams;
Ammonia alum,	6 "
Water,	100 c.c.

Boil for half an hour; add water to make up for that lost by evaporation. Filter and add a crystal of thymol.

Orth's Lithium Carmine.—

Carmine,	2.5 to 5 grams;
Saturated aqueous solution of carbonate of lithium,	100 c.c.;
Thymol,	a crystal.

The carmine dissolves at once in the cold solution. When used as a counter-stain for bacteria in the Gram-Weigert method this solution should be carefully filtered, because organisms occasionally grow in it and may give rise to confusion in the stained preparations.

Neutral Carmine.—Dissolve, without heating, 1 gram of best carmine in 50 c.c. of distilled water plus 5 c.c. of strong aqua ammoniæ. Expose the fluid in an open dish until it no longer smells ammoniacal (about three days); then filter and put away in a bottle for future use. The odor of the solution will soon become bad, but the staining properties will remain unaffected.

Aniline Dyes.—It is extremely important that all aniline dyes used in histological work should be obtained, with possibly a few exceptions, from Grübler, either directly or from his authorized agents. In no other way is it possible to obtain with certainty the results expected. In this country Eimer & Amend, of New York City, are the chief agents for Grübler.

Aniline dyes come in the form of a powder or as crystals, and most of them keep well in that condition. Methylene-blue for one, however, seems to be an exception. After the original package has been opened for a short while the dye is said to lose in intensity of staining power. It is well to keep on hand saturated alcoholic solutions of certain of the dyes, because they keep well in that form, and are ready for use when a saturated alcoholic solution is wanted. This is particularly true of methylene-blue, fuchsin, and gentian-violet.

Aniline dyes are derived from either aniline or toluidin, or from both together. They may be regarded as salts having basic or acid properties. The basic colors stain cell-nuclei, including bacteria, for which they show a marked affinity. The acid colors stain diffusely. The basic dyes most commonly employed in pathological histology are methylene-blue, fuchsin, gentian-violet, and safranin. Of the acid colors, eosin, picric acid, and acid fuchsin are most in use.

As a rule, every aniline dye has one or more standard solutions which are used largely to the exclusion of others, for the reason that, being required for certain purposes, they are kept in stock. As they are thus always at hand, they are used where simple solutions might be used. For instance, Löffler's methylene-blue solution is often used, because ready and convenient, when a simple aqueous solution would do as well.

In the following pages we have arranged under each dye the solutions of it most in use:

Methylene-blue.

1. Saturated solution in 95 per cent. or absolute alcohol. A stock solution to be used in making other solutions. It can be used as a stain by adding 1 part to 9 parts of water.

2. Aqueous solutions of various strengths are often used, and can be made up as needed.

3. *Löffler's Methylene-blue Solution.—*

Saturated alcoholic solution of methylene-blue,	30 c.c.;
Solution of caustic potash in water, 1 : 10,000,	100 "

This is one of the most useful of the aniline staining solutions, and will keep for a long time without losing much in staining power.

4. *Kühne's Methylene-blue Solution.—*

Saturated alcoholic solution of methylene-blue,	10;
5 per cent. carbolic-acid water,	90.

This is a stronger staining solution than Löffler's, but the resulting stain does not seem so sharp and clear.

5. *Gabbet's Methylene-blue Solution.—*

Methylene-blue,	2;
Sulphuric acid,	25;
Water,	75.

It is used as a decolorizer and contrast-stain for tubercle bacilli.

6. *Unna's Alkaline Methylene-blue Solution.*—The strongly alkaline solution of methylene-blue recommended by Unna for staining plasma-cells has been found extremely valuable as a general stain in connection with eosin, which should be used first. The solution should be diluted 1 : 10, or even more, for staining:

Methylene-blue,	1;
Carbonate of potassium,	1;
Water,	100.

7. *Unna's Polychrome Methylene-blue Solution.*—The polychrome methylene-blue solution, much used by Unna in various staining methods, is an old alkaline solution of methylene-blue, of which the above is the original formula, in which, in consequence of oxidation, methyl-violet and methylene-red have formed. Months are required for the process of oxidation to take place. The ripened solution may be obtained from Grübler.

8. *Sahli's Borax Methylene-blue Solution.*—

Saturated aqueous solution of methylene-blue,	24;
5 per cent. solution of borax,	16;
Water,	40.

Mix, let stand a day, and filter.

Fuchsin.

1. Saturated alcoholic solution to be kept in stock.
2. *Ziehl-Neelson's Carbol-fuchsin.*—

Saturated alcoholic solution of fuchsin,	10 c.c.;
5 per cent. carbolic-acid water,	90 "

This solution is very powerful, stains quickly, keeps well, and can be employed for a variety of purposes.

3. *Aniline-fuchsin.*—

Saturated alcoholic solution of fuchsin,	16 c.c.;
Aniline-water,	84 "

Gentian-violet.

1. Saturated alcoholic solution to be kept in stock.
2. *Ehrlich's Aniline-gentian-violet.*—

Saturated alcoholic solution of gentian-violet,	16 c.c.;
Aniline-water,	84 "

During the first few hours after the solution is made considerable precipitation takes place, so that it is best not to use it for twenty-four hours. After about ten days it begins to lose its staining power.

Zenker recommends a solution without alcohol: Dissolve the gentian-violet directly in the aniline-water. The color is said to be less easily removed from tissues when this solution is used.

3. *Stirling's Solution of Gentian-violet.*—

Gentian-violet,	5 grams;
Alcohol,	10 c.c.;
Aniline,	2 "
Water,	88 "

This solution is said to keep remarkably well.

4. *Carbol-gentian Violet.—*

Saturated alcoholic solution of gentian-violet,	10 c.c.;
5 per cent. carbolic-acid water,	90 "

Safranin.—Two of the many preparations by this name have been found especially useful:

1. Safranin O soluble in water.
2. Safranin soluble in alcohol.

The three following solutions of safranin can be thoroughly recommended:

1. Saturated aqueous solution of "safranin O soluble in water" (to be made with the aid of heat).

2. A mixture of equal parts of—

A saturated aqueous solution of "safranin O soluble in water."

A saturated alcoholic solution of "safranin soluble in alcohol."

3. *Babes' Aniline Safranin.—*

2 per cent. aniline-water,	100;
"Safranin O soluble in water,"	in excess.

Saturate the solution by heating it in a flask set in hot water to 60–80° C.; filter.

This solution is extremely powerful, stains almost instantly, and will keep about two months.

Methyl-violet.—1. Aqueous solutions of various strengths, $\frac{1}{2}$ to 2 per cent., keep well and are used for staining nuclei, bacteria, and amyloid.

2. Methyl-violet can be used instead of gentian-violet in Ehrlich's solution.

3. For staining neuroglia-fibers Weigert employs a saturated solution made with the aid of heat in 70–80 per cent. alcohol.

Bismarck Brown.—The most common solutions are the following:

1. A 1 per cent. aqueous solution.

2. A saturated aqueous solution made by boiling (3–4 per cent.).

3. A saturated solution in 40 per cent. alcohol (2–2½ per cent.).

Unlike other aniline colors, Bismarck brown will keep in glycerin mounts and can be fixed in nuclei by acid alcohol. The stain is not used so much as formerly, except as a contrast stain in Gram's method and for photographic purposes. Other basic stains less frequently used, and then generally in aqueous solutions, are dahlia, methyl-green, iodin-green, and thionin.

Diffuse Stains.—1. **Eosin** is sold in two forms—as "eosin soluble in water," and as "eosin soluble in alcohol." The first is to be preferred, because a greater degree of differentiation in stain can be obtained with it. Keep on hand a saturated aqueous solution and dilute with water as needed. The strength of solution to be used varies somewhat with the tissue and the reagent in which it has been fixed, but generally lies between $\frac{1}{10}$ and ½ per cent. when the eosin is used after a hematoxylin stain. These dilute solutions should contain 25 per cent. of alcohol, otherwise they will not keep well. When eosin is employed before an aniline dye such as methylene-blue, a 5 per cent. or even a saturated solution should be taken.

2. **Picric Acid.**—Saturated alcoholic and aqueous solutions should be kept in stock, to be diluted as needed.

3. **Van Gieson's Picro-fuchsin Solution.**—This valuable solution was originally made by adding to a saturated aqueous solution of picric acid enough of a saturated aqueous solution of acid fuchsin to give to the fluid a deep garnet-red color, and for certain purposes, as in staining after Zenker's fluid, this strong solution is to be preferred. Freeborn has recently given more precise directions for making up the solution according to the purpose for which it is to be used.

For Connective Tissue.—(See page 303).

1 per cent. aqueous solution of acid fuchsin,	5 c.c.;
Saturated aqueous solution of picric acid,	100 "

For the Nervous System.—(See page 311).

1 per cent aqueous solution of acid fuchsin,	15 c.c.;
Saturated aqueous solution of picric acid,	50 "
Water,	50 "

Picro-nigrosin (*Martinotti*).—Dissolve picric acid and nigrosin to saturation in 70 per cent. alcohol.

Combination Stains.—**Biondi-Heidenhain Staining Solution.**—

Saturated aqueous solution of orange G,	100;
Saturated aqueous solution of acid fuchsin,	20;
Saturated aqueous solution of methyl-green,	50.

Make up the separate solutions and let them stand for several days with excess of coloring matter (shaking the bottles occasionally) until they are saturated. Then mix the solutions. For staining dilute the combined solution with water 1 : 100.

The following tests are used for finding out if the proper combination has been obtained: The addition of acetic acid should make the solution redder; a drop of the solution on filter-paper should make a blue spot with green in the center and orange at the periphery. If a red zone appears outside of the orange, then too much acid fuchsin is present.

Pianese's Staining Solutions and Staining Methods.—The following stains, devised by Pianese, are recommended by him particularly for the study of cancer, but will be found useful in many lines of histological investigation. The first two were used by him for tissues hardened in corrosive sublimate or in Zenker's fluid; the others, only after his special fixative (given on page 249). The methods are intended for paraffin sections:

I. Carmine and Picro-nigrosin.—1. Stain in neutral or lithium carmine.

2. Decolorize in acid alcohol.
3. Wash in water.
4. Absolute alcohol.
5. Aniline-gentian-violet, ten minutes.

6. Iodin solution, two to three minutes.
7. Absolute alcohol, so long as any color is discharged.
8. Saturated aqueous solution of picric acid and of nigrosin, five minutes.
9. Decolorize in a 1 per cent. alcoholic solution of oxalic acid.
10. Water, several minutes.
11. Absolute alcohol.
12. Oil of bergamot.
13. Balsam.

Nuclei, red; cell-protoplasm, light olive-green; connective tissue, dark olive-green; elastic fibers, bluish; bacteria and blastomycetes, violet.

II. **Methylene-blue and Eosin in Borax Solution.**—Keep three solutions on hand:

(*a*) Saturated solution of methylene-blue in a saturated aqueous solution of borax.

(*b*) ½ per cent. solution of "bluish eosin" in 70 per cent. alcohol.

(*c*) Saturated aqueous solution of borax.

For use mix together 2 parts of the filtered solution *a*, 1 of *b*, and 2 of *c*. The different steps of the staining process are as follows:

1. Absolute alcohol.
2. Staining solution, ten to twenty minutes.
3. Decolorize in a 1 per cent. solution of acetic acid.
4. Wash in water.
5. Absolute alcohol.
6. Xylol.
7. Xylol balsam.

Nuclei, blue; red blood-globules, cell-protoplasm, granules of eosinophiles, connective tissue, etc., rose-red.

III. *a*. **Malachite-green, Acid Fuchsin, and Nigrosin.**—

Malachite-green,	1.	gram;
Acid fuchsin,	.4	"
Nigrosin,	.1	"
Water,	50	c.c.;
Alcohol saturated with acetate of copper,	50	"

1. Absolute alcohol.
2. Stain in 20 drops of above solution diluted with 10 c.c. of distilled water for twenty-four hours.
3. Decolorize in a ½ per cent. aqueous solution of oxalic acid.
4. Wash in water.
5. Absolute alcohol.
6. Xylol balsam.

Resting nuclei, light red; protoplasm, reddish yellow. In the karyokinetic figures, nuclein green; fibrillæ of the achromatic spindle and of the mitoma, bright red; centrosome and polar bodies, red; the rest of the cell-body, a reddish-yellow color.

III. *b.* **Malachite-green, Acid Fuchsin, and Martius Yellow.**—

Malachite-green,	.5	gram;
Acid fuchsin,	.1	"
Martius yellow,	.01	"
Distilled water,	150	c.c.;
Alcohol, 96 per cent.,	50	"

1. Stain in the solution without diluting, half an hour.
2. Absolute alcohol.
3. Xylol.
4. Xylol balsam.

Nuclei of resting and dividing cells, green; cell-protoplasm, connective tissue, etc., rose-colored; "cancer-bodies," mainly red, but in masses of them some are red and some green.

IV. **Acid Fuchsin and Picro-nigrosin.**—

Saturated alcoholic solution of acid fuchsin,	6	drops;
Martinotti's picro-nigrosin,	8	"
Distilled water,	10	c.c.

1. 70 per cent. alcohol.
2. Stain in the solution six hours.
3. Decolorize in dilute acetic acid.
4. Absolute alcohol.
5. Xylol.
6. Xylol balsam.

Resting nuclei, red; nuclein of karyokinetic figures, yellow: cell-protoplasm, dark olive-green; "cancer-bodies," mainly olive-gray, but some or portions of them may be ruby-red.

V. **Light Green (Lichtgrün) and Hematoxylin.**—

Ehrlich's acid hematoxylin,	15 c.c.
Saturated solution of Lichtgrün in 70 per cent. alcohol,	5 "
Distilled water,	15 "

1. Distilled water.
2. Stain in above mixture half an hour.
3. Wash thoroughly in several waters.
4. Alcohol.
5. Oil of bergamot.
6. Balsam.

Nuclei, green; "cancer-bodies" take the hematoxylin stain.

VI. **Acid Fuchsin and Hematoxylin.**—

Ehrlich's acid hematoxylin,	15 c.c.
1 per cent. solution of acid fuchsin in 70 per cent. alcohol,	15 "
Distilled water,	15 "

Stain as in V.

Nuclei, red; protoplasm, brick-red; "cancer-bodies" take the hematoxylin stain.

Orcein, a vegetable dye obtained from certain tinctorial lichens, is used mainly for staining elastic fibers. It is soluble in alcohol, and is employed either in a neutral or acid (HCl) alcoholic solution.

Iodin is the oldest of the histological stains, but is now but little used for that purpose.

The *tincture of iodin,* a saturated solution in alcohol, is used for getting rid of the precipitate of mercury formed in tissues fixed in corrosive sublimate or in Zenker's fluid.

Lugol's solution, a solution of iodin in water containing iodid of potash, is of varying strength. Iodin in this form is much used as a test for starch, amyloid, glycogen, and corpora amylacea. In Gram's stain and its modifications iodin produces some chemical change in the coloring material employed, in consequence of which, when appropriate decolorizers are used, the stain remains fast in certain structures, while from others it is easily entirely extracted.

The strength originally employed by Gram for his staining method was—

Iodin,	1 gram;
Iodid of potash,	2 grams;
Water,	300 c.c.

Weigert in his modification of this method employed a stronger solution:

Iodin,	1 gram;
Iodid of potash,	2 grams;
Water,	100 c.c.

Recently he has recommended the following strength both for fibrin and for neuroglia-fibers:

Iodid of potash,	5 grams	saturated with iodin.
Water,	100 c.c.	

The only difference in the action of the various solutions probably is that the strong solution acts practically instantaneously, while the weaker solutions require some little time.

Acid Alcohol (*Orth's Discharging Fluid*).—

Hydrochloric acid,	1 c.c.;
70 per cent. alcohol,	99 "

Aniline Water (*Aniline-oil Water*).—Shake together 5 parts of aniline with 95 parts of water, and filter the resulting milky fluid. It should come through perfectly clear.

Carbolic-acid water is made in like manner by shaking together 5 c.c. of melted carbolic-acid crystals and 95 c.c. of water. The solution should be filtered.

Mayer's glycerin-albumin mixture for attaching paraffin sections to slides is composed of equal parts of the white of egg and of glycerin. The mixture should be thoroughly beaten and then filtered, or after standing for some time can be decanted. Add a little camphor or carbolic acid to prevent decomposition. Egg-albumin is dissolved by acids and alkalies, so that when such reagents are to be used the sections are best attached to the slide by some other substance. For this purpose *Schällibaum's solution*, of celloidin 1 part in 3 or 4 parts of oil of cloves, is often useful. Cover the slide with a thin layer of the solution. Arrange the sections in order on the slide and place it in the thermostat at 54° to 60° C. for several hours, or heat for a few seconds to half a minute over the flame until the oil of cloves runs together in drops. After cooling, remove the paraffin with xylol, pass through origanum oil to 95 per cent. alcohol, and proceed as with other paraffin sections.

Clearing Reagents.—The object of clearing reagents is to render certain tissue-elements more prominent than others. This result may be brought about by dilute acetic acid (2–5 : 100), which swells up the ground substance, so that nuclei, elastic fibers, fat, myelin, and micro-organisms are more distinct, or by alkalies, which destroy the cells and ground substance and leave only elastic fibers and bacteria but little changed. This method is used almost wholly for fresh tissues.

The same result is more commonly obtained by soaking the tissues in substances which by reason of their high index of refraction render the tissues more or less transparent. Any structure which it is desirable to study is usually previously stained and thus easily rendered prominent. This second method is most applicable to hardened tissues.

For soaking and clearing the tissues a variety of reagents of different chemical properties are used. Glycerin and acetate of potash are not so much employed as formerly, because balsam mounts are more generally preferred. Of the other reagents (ethereal oils and coal-tar products), the the choice depends mainly on two factors—the kind of stain which has been employed, and the substance in which the sections have been imbedded. Many of the clearing reagents either dissolve celloidin or will not clear it from 95 per cent. alcohol, and nearly all of them will extract aniline colors more or less rapidly.

Most of the clearing reagents can be used after hematoxylin and carmine stains. For celloidin or paraffin sections stained by either of them oleum origani cretici, oil of bergamot, or the mixture of the oils of cloves and thyme is recommended in the order given.

For aniline stains the best clearing reagent is xylol, which, however, clears directly only from absolute alcohol. It can be used, however, for celloidin or other sections dehydrated in 95 per cent. alcohol by a simple method original with Welch, and lately brought into notice by Weigert. Blot the section on the slide with smooth soft filter-paper, and then pour on a few drops of xylol; repeat the blotting, followed by xylol two or three times, and the section will be found to be perfectly clear.

Oleum Origani Cretici.—Colorless to light brown in color; clears readily from 95 per cent. alcohol without dissolving celloidin; affects aniline colors slowly. Ordinary origanum oil is impure oil of thyme, and should not be used.

Oil of Bergamot.—Light green in color; clears quickly from 95 per cent. alcohol; does not dissolve celloidin, but after repeated use of the same lot of oil it will sometimes soften it a little. Affects aniline colors slowly, with the exception of eosin, which it extracts very quickly.

Oil of Cloves.—Straw-colored; clears quickly from 95 per cent. alcohol; dissolves celloidin; extracts aniline colors, especially methylene-blue.

Oil of Thyme.—Colorless; clears readily from 95 per cent. alcohol; makes sections brittle; does not dissolve celloidin; affects aniline colors.

Oil of Lavender.—Clears celloidin sections readily from 95 per cent. alcohol.

Oil of Cedar-wood.—Pale straw-color; clears from 95 per cent. alcohol, but, unfortunately, clears celloidin sections very slowly; does not affect aniline colors.

Aniline (*Aniline Oil*).—Colorless when perfectly pure and fresh, but soon oxidizes and turns brown; does not dissolve celloidin; clears readily from 70 per cent. alcohol; will clear from water by Weigert's method; extracts aniline colors slowly.

Xylol.—Colorless; does not dissolve celloidin; does not affect aniline colors; clears directly only from absolute alcohol; but will clear even celloidin sections from 95 per cent. alcohol if they be blotted on the slide, and the xylol be then poured over them; the process of blotting followed by xylol must be repeated two or three times.

Dunham's Mixture of the Oils of Cloves and Thyme.—Excellent for sections stained in hematoxylin or carmine. Not nearly so expensive as pure origanum or bergamot oil.

Oil of cloves,	1 part;
Oil of thyme,	4 parts.

Filter if cloudy; clears celloidin sections readily from 95 per cent. alcohol without dissolving the celloidin.

Weigert's Mixture of Carbolic Acid and Xylol.—

Carbolic-acid crystals,	1 part;
Xylol,	3 parts.

Recommended for clearing thick sections of the central nervous system after carmine and hematoxylin stains only. The next mixture is more used now-a-days.

Weigert's Mixture of Aniline and Xylol.—

Aniline,	2 parts;
Xylol,	1 part.

Mounting Reagents.—The most generally used reagent for permanent mounts is Canada balsam. Damar and colophonium are only exceptionally preferred.

Canada balsam occurs in commerce as a very thick, tenacious, pale, straw-colored fluid. It should be evaporated over a water-bath to drive off all volatile substances, which might affect aniline colors, until it becomes solid and brittle on cooling. Dissolve it then in xylol, which does not affect aniline colors, to a rather thin, syrupy consistency. Two pounds of Canada balsam will evaporate to about one pound; add xylol enough to make the mixture up to two pounds. In this condition it is often called *xylol balsam.*

Canada balsam has a high index of refraction, so that tissues mounted in it become very transparent, and only those parts are visible which are stained. Other solvents of Canada balsam, such as chloroform and benzine, may be used, but cannot be recommended for sections stained with aniline colors. For tissue stained with osmic acid, *chloroform balsam*, prepared in the same way as xylol balsam, should always be used, otherwise the osmic acid stain will fade rapidly.

Damar has a lower index of refraction than Canada balsam; is soluble in xylol, chloroform, etc.; dries slowly, and is generally recommended for Golgi preparations.

Colophonium dissolved in benzine is employed by Nissl for mounting stained preparations of ganglion-cells.

METALLIC STAINS OR IMPREGNATIONS.

EXPERIMENTAL investigation has shown that certain metals can be used for staining certain tissue-elements, either because they are directly reduced from solutions of appropriate salts or because they are taken up and retained by certain tissue-elements, which are rendered prominent when the metallic salt is reduced later. The most valuable metals for this purpose are silver, gold, and osmium.

Silver is used, generally in the form of silver nitrate, to stain of a brown or dark-brown color the cement substance between epithelial and endothelial cells and the ground substance of connective tissue. The method finds its chief use in pathology in demonstrating the endothelial covering of a doubtful surface, in outlining the endothelial cells of pathologically altered blood- and lymph-vessels, and in staining the ground substance of the connective tissue of the cornea when that organ is used experimentally for the study of inflammation. In combination with certain other salts, especially bichromate of potassium, nitrate of silver is much employed in the Golgi methods to stain ganglion-cells and their processes in the central nervous system.

The difficulty of the silver method lies in the fact that the salt forms with albuminous fluids granular and thread-like coagula which can easily give rise to false pictures. For this reason the method is limited almost entirely to natural surfaces, which should be washed off with water or a 2 per cent. solution of nitrate of sodium before the silver solution is applied. It is generally advisable to use the nitrate of silver in a very dilute solution, 1 : 250 or 500. The solution is allowed to act on the surface for about a minute, and is then washed off with water. The tissue is next exposed in water to the action either of sunlight or of diffuse light. The outlines of the cells soon appear as dark lines, brown to black in color. The tissue to be stained should be kept stretched, because a precipitation of the silver occurs wherever there is a fold in the surface. Although nitrate of silver penetrates but a slight distance, it is possible to stain the outlines of the endothelial cells of the lymphatics and blood-vessels as well as the ground substance of the connective tissue—in a rabbit's diaphragm, for instance—by treating the upper or lower surface with the silver solution. The thoracic organs should be removed, and then the upper surface of the tendinous portion of the diaphragm left *in situ* is exposed to the action of the silver salt in the manner already described.

The outlines of the endothelial cells of blood-vessels are usually stained by injections of the silver salt through an artery. In the same way the limits of the epithelial cells of the alveoli of the lung can be stained by injections through a bronchus.

Although generally employed in solution, nitrate of silver is sometimes used in the solid form, and for the cornea this method is preferable. Chloroform the animal, preferably a rabbit, deeply; rub the cornea with a stick of nitrate of silver hard enough to remove the surface epithelium. Allow the salt to act about ten minutes, then kill the animal, remove the eye, cut out the cornea, wash it, and expose to diffuse daylight for half an hour. It is then placed in a mixture of glycerin and water, 30 parts to 70, very slightly acidulated with acetic acid (about $\frac{1}{10}$ per cent.) for twenty-four hours, so as slightly to swell and to soften the tissues. Sections of the cornea are best made with the freezing microtome. Incise the periphery a little at four points equally distant from each other, so that the cornea will lie flat. A direct stain with alum-hematoxylin gives by all odds the best results. The sections may be mounted in glycerin or balsam. The latter method is perhaps the better. Dehydrate the sections in 50 per cent., then in 70 per cent., alcohol, clear in aniline oil, wash with xylol, and imbed in balsam. This method avoids the shrinkage which is caused by using strong alcohol.

Gold, in the form of the simple or double chlorid, is employed to stain the protoplasm of cells of connective tissue, and more particularly the axis-cylinders of nerve-fibers and their terminal processes. Like nitrate of silver, it acts as a fixing and hardening reagent as well as a stain. Unfortunately, it penetrates tissues but a very slight distance, and, so far as staining is concerned, is inconstant in action. Its chief use in pathology is in connection with experimental work on the cornea and in regeneration. The conditions under which the reduction of the gold salt takes place are not exactly understood, but both penetration and reduction are aided by the action of organic acids, such as formic, citric, and tartaric acids, on the tissues both before and after the treatment with the gold salt. Of the many methods proposed, the following are recommended:

Löwit's Formic-acid Method.—1. Place very small bits of fresh tissue in a mixture of formic acid 1 part, and water 1 to 2 parts, until they become transparent (a few seconds to several minutes).

2. Transfer to chlorid of gold, 1 to 1.5 parts to 100 of water, for fifteen minutes.

3. Formic acid, 1 part to water 3 parts, for twenty-four hours.

4. Concentrated formic acid twenty-four hours. Preserve in glycerin or balsam.

All the steps except the first should be performed in the dark.

Ranvier's Formic-acid Method.—1. Boil together 8 c.c. of a 1 per cent. solution of chlorid of gold and 2 c.c. of formic acid. When the solution is cold place very small bits of tissue in it for one hour, in the dark.

2. Wash quickly in water.

3. Expose to diffuse light in a mixture of formic acid 10 c.c. and water 40 c.c. Reduction takes place slowly (twenty-four to forty-eight hours).

4. Harden in 70 per cent., then 90 per cent., alcohol in the dark.

Osmic Acid (perosmic acid, osmium tetroxid) is used as a fixing reagent and for staining fat and myelin, by which it is reduced. As osmic acid is quickly reduced by organic substances, care must be taken in making up the solution. Remove the label from the sealed tube in which the acid comes, and place the tube, after cracking off one end, in a glass-stoppered bottle containing enough water to make a 2 per cent. solution. If desired, the tube can be broken after it is in the bottle by violent shaking. It should be borne in mind that osmic acid is very-irritating to the bronchial mucous membrane.

In a 1 or 2 per cent. solution osmic acid is used to stain fat in teased preparations or frozen sections of fresh tissues. In Marchi's method it is used to stain fat in tissues which have been hardened for some time in Müller's fluid. As a fixing reagent it is usually combined with other reagents, as in Flemming's solution, both for its property as a fixative and for the purpose of staining any fat present.

Preparations stained in osmic acid may be kept indefinitely in alcohol. When sections are mounted they should be cleared in chloroform, and preserved in chloroform balsam prepared in the manner described elsewhere. Xylol and other clearing reagents cause the stain to fade.

STAINING METHODS.

The purpose of staining is to render prominent the different tissue-elements, so that they may be readily recognized and studied. The constant tendency now-a-days is toward selective or differential staining methods, by which but one tissue-element will be colored to the exclusion of all others, or at least of any element that might be confused with it morphologically. These selective stains, which really are micro-chemical color reactions, enable us to differentiate from each other with ease and accuracy cellular and intercellular elements, or pathological products which otherwise look alike.

The list given on page 288 does not pretend to be either complete or perfect in arrangement, but will give some idea of the various elements which we wish to stain. Those for which we now possess more or less perfect differential stains are printed in *italics*.

The simplest selective stain is, of course, that for nuclei, and it can be obtained with a great variety of staining reagents. The most difficult element to stain differentially, although it can be done under certain conditions with a fair amount of success, is probably the axis-cylinder and its terminal processes.

Tissue-elements and pathological products differ from each other, not only in form and consistency, but also in chemical properties. While perfect preservation of form is sufficient to distinguish certain cells or elements from each other—as, for instance, polynuclear leucocytes from lymphoid cells—differentiation based on micro-chemical tests is always to be preferred when possible. A few of the tests employed are colorless, like the precipitation of mucin by acetic acid. Certain tests, like the methylene-blue or gold stain for axis-cylinders, can be applied to fresh tissues only.

Others, like the various amyloid reactions, can be obtained with fresh or hardened tissues. Most of the micro-chemical reactions, however, can be employed only with tissues which

- Cell.
  - Nucleus.
    - *Nucleolus.*
    - *Resting nucleus.*
    - *Linin.*
    - Bacteria.
      - 1. Do not stain by Gram.
      - 2. *Stain by Gram.*
      - 3. *Stain by tubercle-bacillus method.*
    - *Nucleus of ameba coli.*
  - Protoplasm.
    - Centrosome and polar bodies.
    - Granules.
      - *Mastzellen.*
      - *Plasma-cell of Unna.*
      - Leucocytes.
        - *Five kinds of granules* described by Ehrlich.
      - *Nissl's granules in ganglion-cells.*
    - *Dendritic processes of ganglion-cells.*
    - *Axis-cylinder and terminal processes.*
    - *Contractile elements of striated muscle-fiber.*
    - *Red blood-globules.*
    - *Cilia of bacteria.*
  - Cuticle.
    - *Certain dots or lines in ependymal cells.*
    - *So-called cilia in certain renal cells.*
    - *Bile-capillaries.*

- Intercellular substances.
  - *Cement substance of epithelial and endothelial cells.*
  - *Ground substance of connective tissue.*
  - *Connective-tissue fibrillæ and reticulum.*
  - Myxomatous tissue; *mucin.*
  - *Elastic fibers.*
  - Intercellular substances of cartilage.
  - Ground substance of bone.
  - *Myelin.*
  - *Neuroglia-fibers.*
  - *Clubs of actinomyces.*
  - *Capsules of bacteria.*

- Pathological products.
  - Hyaline substances.
    - *Fibrin.*
    - *Mucin.*
    - *Amyloid.*
    - *Glycogen.*
    - Hyalin.
    - Colloid.
    - *Keratohyalin.*
    - *Eleadin.*
  - *Fat.*
  - *Hemosiderin.*
  - Hematoidin.
  - *Hemoglobin.*

have been properly preserved. It is exceedingly important, therefore, that a tissue-element be so fixed and hardened that its peculiar chemical properties be preserved intact, otherwise a differential stain for it is impossible. Each tissue-element is a law unto itself. For example, the peculiar chemical properties of red blood-globules depend on the presence in them of hemoglobin. As a differential stain of the red blood-globules depends on fixing this substance in them, it is necessary to find out the chemical properties of hemoglobin, such as the fact that it is soluble in water or dilute alcohol, but not in salt solution, and that it is fixed in the red blood-globules by heat, absolute alcohol and ether equal parts, corrosive sublimate, formaldehyde, bichromate of potassium, etc.

While differential stains depend in part on the chemical properties of the tissue-elements, they also depend to a certain extent on the chemical properties of the staining reagents and the decolorizers used.

Some of the tissue-elements can be stained differentially in a number of ways, sometimes after one fixing agent, sometimes after another. The simplest differential stains are those where certain tissue-elements stain directly in a given solution after they have been properly fixed. Good examples are—Ehrlich's triple stain for certain protoplasmic granules in leucocytes, and the direct stain for elastic fibers with an acid alcoholic solution of orcein.

Other differential stains depend on the property of certain elements to hold colors they have once taken up when treated with decolorizers. The best example of this is the tubercle bacillus, which holds certain stains through various acids, or aniline hydrochlorate, followed by alcohol, and, if necessary, by a contrast-stain.

Still another varied group of elements (certain bacteria, fibrin, neuroglia-fibers, etc.) depends for a differential stain in part on changes produced in gentian- or methyl-violet by iodin, in part on the decolorizer employed for extracting the coloring reagent.

Although the steps of the various staining methods differ considerably, they may be roughly arranged in the following order:

1. Staining.
2. Differentiating.
3. Decolorizing.
4. Dehydrating.
5. Clearing.
6. Mounting.

Very often two or more of the steps are combined in one, as when aniline oil is used for decolorizing, dehydrating, and clearing sections stained for certain bacteria. Sometimes the staining process occupies more than one step, as in Weigert's myelin-sheath stain. In alum-hematoxylin the differentiating reagent, the excess of alum, is combined with the stain; in Gram's method the differentiating reagent, iodin, forms a step by itself.

NUCLEAR STAINS.

For general histological work few stains are more valuable or can be more highly recommended than alum-hematoxylin, either alone or in contrast with eosin. Properly made and used, the solution stains the nuclei sharply and of varying degrees of intensity, depending on the character of the cells. Besides the nuclei, however, it stains other tissue-elements in delicate shades of blue, so that they are readily visible, and thus more or less differentiated from those structures which fail to stain.

Of the carmine stains, lithium carmine, followed by picric acid, will be found the most brilliant, generally useful, and permanent.

Safranin gives, perhaps, the most permanent stain of any of the basic aniline dyes, and confines itself very sharply to the nuclei. It is much used after certain fixing reagents, such as Flemming's and Hermann's solutions. Eosin, followed by methylene-blue, gives beautiful results, especially when Unna's alkaline solution of methylene-blue is used. The advantages of this solution are that it stains readily tissues hardened in Zenker's fluid and brings out nuclei and nuclear figures with great sharpness, while at the same time it stains the protoplasm of certain cells so that they are easily distinguished from other cells. The Heidenhain-Biondi triple stain is useful after fixation in corrosive sublimate, but cannot be employed with celloidin sections, so that its field is limited. The other aniline dyes are used on occasion or for some definite purpose, but not so generally as those mentioned above.

Alum-hematoxylin Stains.—Most alum-hematoxylin solutions will over-stain if the sections are left too long in them. The proper time required depends on the fixing reagent used and on the degree of ripeness of the staining solution. It is therefore advisable to wash a section in water occasionally and decide from the color it has acquired if it be sufficiently stained, or to mount it in water on a slide and examine with the low power of the microscope.

The best results are obtained with alum-hematoxylin solutions by staining sections just deeply enough, washing them thoroughly in several changes of water, and leaving them in a large dish of water over night. This thorough washing is done to rid the tissues of every trace of alum or of acid, so that the color will become a clear blue and will keep indefinitely.

Many microscopists prefer to stain deeply and diffusely in an old, quickly-staining alum-hematoxylin solution, and then to employ a decolorizer. The agents most used for the purpose are alum (1 per cent. aqueous solution for one to two hours), hydrochloric acid ($\frac{1}{10}$ to $\frac{1}{2}$ per cent. aqueous solution, or even the ordinary acid alcohol), and acetic acid (1 to 3 per cent. solution) for a few seconds only. After being sufficiently decolorized the sections must be thoroughly washed in water, preferably for a number of hours, otherwise the stain will fade. The objection to this method is that a pure nuclear stain only is obtained, because the acid removes the color completely from all the rest of the tissue. Under certain circumstances, as when hematoxylin is used as a contrast-stain to fuchsin in staining for tubercle bacilli, such a sharp limitation to the nuclei is desirable.

Alum-hematoxylin stains well and quickly tissues hardened in alcohol, in corrosive sublimate, and in picric acid. It stains much more slowly tissues hardened in solutions containing chrome salts, such as Zenker's and Müller's fluids.

For counter-staining eosin will usually be found to give the most beautiful contrast, although picric acid, Van Gieson's mixture, and neutral carmine are often of service.

A good alum-hematoxylin solution should have a bluish or purplish color, and should stain celloidin very faintly or not at all.

Aqueous Alum-hematoxylin; Delafield's Hematoxylin; Ehrlich's Acid Hematoxylin (see pages 265 and 266).

1. Stain in one of the above solutions two, five, or thirty minutes, or sometimes even longer.

2. Wash in several changes of water, and then leave sections, if possible, for several hours or over night in a large dish of water; or better still, wash in running tap water for ten to thirty minutes.

3. Contrast-stain, usually an aqueous solution of eosin, $\frac{1}{10}$ to $\frac{1}{2}$ per cent., for one to five minutes.

4. Alcohol, 95 per cent., two or three changes to dehydrate and to remove excess of contrast-stain.

5. Clear in oleum origani cretici or in Dunham's oils-of-cloves-and-thyme mixture.

6. Canada balsam.

The staining of the nuclei by Ehlrich's alum-hematoxylin solution is not so sharp as that obtained by the simple aqueous solution.

The more customary method of using Delafield's alum-hematoxylin solution is to filter a few drops of it into a dish of water and to stain sections for a long time, even over night, with the very dilute solution thus obtained. It is sometimes advisable to use the aqueous solution in the same way.

Mayer's Hemalum (see page 266).—1. Stain three to five minutes or longer.

2. Wash out in 1 per cent. alum solution until the stain is precise.

3. Wash thoroughly in several changes of water.

4. Alcohol, 95 per cent.

5. Oleum origani cretici.

6. Canada balsam.

The staining is rather diffuse, so that it has to be washed out to some extent with alum-water. Mayer's acid hemalum is more precise, and usually does not need to be decolorized, so that the second step can be omitted.

Hemalum is used for staining tissues in bulk. Twenty-four hours are required for large pieces.

Heidenhain's Hematoxylin Stain.—1. Stain twenty-four to forty-eight hours in a simple ½ per cent. aqueous solution of hematoxylin dissolved by the aid of heat.

2. Transfer the sections directly to a ⅓ per cent. aqueous solution of simple chromate of potassium for twenty-four to forty-eight hours, changing the solution frequently until no more color is given off by the sections.

3. Wash thoroughly in water.

4. Alcohol.

5. Oil.

6. Canada balsam.

Mallory's Chlorid of Iron Hematoxylin.[1]—The results which can be obtained by this method are equally quick and satisfactory after all of the usual fixing reagents except, perhaps, formaldehyde.

Celloidin or paraffin can be employed for embedding.

1. Stain sections on the slide for three to five minutes in a 10 per cent. aqueous solution of ferric chlorid.

2. Drain and blot the sections; then pour over them a few drops of a freshly prepared 1 per cent. aqueous solution of hematoxylin. If all of the hematoxylin is precipitated by the excess of ferric chlorid, pour off the solution and add a fresh supply. In three to five minutes the sections will be colored a dark bluish-black.

3. Wash in water.

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[1] Mallory: *The Journal of Experimental Medicine*, 1900, v., 18.

4. Decolorize and differentiate in a ¼ per cent. aqueous solution of ferric chlorid. The sections should be kept constantly moving in the solution. The differentiation will be complete in a few seconds to one or more minutes.
5. Wash in water.
6. Dehydrate in alcohol.
7. Clear in oleum origani cretici.
8. Xylol balsam.

In the above directions definite strengths have been assigned to the solutions, but they may vary greatly without affecting the result. The important point is to get the sections stained deeply, and then to decolorize slowly. The differentiation can be stopped at any moment by transferring the sections to water. Sometimes it is advisable to examine the sections under the microscope to see if enough color has been extracted.

The strength of the hematoxylin solution is unimportant; it is simply necessary to have enough hematoxylin to combine with all of the iron in and on the section. The simplest way is to dissolve by the aid of heat a pinch of the crystals in a few cubic centimeters of water. A little experience will determine about how much is needed. If a solution of hematoxylin more than one or two days old is used, the color obtained is grayish-blue, and not so bright.

This method gives a sharp, permanent, dark-blue stain to nuclei; it also stains fibrin of a grayish to dark-blue color; if the decolorization is not carried too far, the contractile elements of striated muscle are brought out very sharply. In Zenker preparations the red globules appear of a greenish-gray color. Connective tissue is tinted a pale yellow. The nucleus of the amœba coli stains sharply by this method.

Carmine Stains.—The ordinary carmine solutions give good nuclear stains, but of the finer details in a specimen they bring out much less than a direct alum-hematoxylin stain. They are much less used now than formerly, except as contrast-stains to bacteria and to fibrin in the methods of Gram and Weigert, for which purpose lithium carmine will usually give the best results.

Alum Carmine; Alum Cochineal (see page 269).—1. Water.

2. Stain in either of the above solutions for five to twenty minutes.

3. Wash thoroughly in water.

4. Alcohol, 95 per cent.

5. Oleum origani cretici.

6. Canada balsam.

Over-staining does not occur. The solutions cannot be recommended for tissues which stain with difficulty. When used for staining in bulk, twenty-four to forty-eight hours are required.

Lithium Carmine (see page 269).—1. Water.

2. Stain two to five minutes.

3. Transfer directly to acid alcohol, one or more changes for several minutes or more, until the sections are well differentiated.

4. Wash in water.

5. Alcohol, 95 per cent.

6. Oleum origani cretici.

7. Canada balsam.

This method gives an intense and permanent bright-red nuclear stain. Over-staining is impossible. A trace of picric acid added to the alcohol used for dehydration affords a beautiful contrast-stain.

Aniline Dyes as Nuclear Stains.—Any of the basic aniline dyes may be used as nuclear stains after the following general method:

1. Stain paraffin sections in a strong solution of the dye preferred in water or in dilute alcohol for five to thirty minutes.

2. Wash in water.

3. Dehydrate in absolute alcohol.

4. Clear in xylol.

5. Xylol balsam.

With celloidin sections use 95 per cent. alcohol, blot with filter paper, and clear in xylol.

As a matter of fact, however, certain dyes and certain solutions are generally used in preference to the others. Most of the colors are more or less affected by all clearing reagents except xylol. With paraffin sections and those from which the celloidin has been removed it is very easy to dehydrate in absolute alcohol and to clear in xylol. With celloidin sections, however, this is impossible, because the absolute alcohol will dissolve out the celloidin, and this is usually not desirable. For celloidin sections, therefore, blot with filter paper, and then pour on xylol; repeat the blotting, followed by xylol, two or three times until the specimen is perfectly clear. Mount in xylol balsam.

In washing out the excess of color it is sometimes found advantageous to acidulate very slightly either the water or the first alcohol with acetic or hydrochloric acid. This process, if not carried too far, tends to make the nuclear stain sharper.

Safranin is one of the very best nuclear-staining aniline dyes. Tissues may be hardened in alcohol, corrosive sublimate, Flemming's, Hermann's, or Zenker's fluids. Any one of the solutions of safranin given on page 273 may be used.

1. Stain paraffin sections two to five minutes to twenty-four hours according to the staining solution and fixing reagent used.
2. Wash in water.
3. Absolute alcohol, several changes, until the section appears properly differentiated.
4. Xylol.
5. Xylol balsam.

For celloidin sections dehydrate in 95 per cent. alcohol, clear in bergamot or origanum oil, and wash out in xylol. To render the stain more precise a few drops of acid alcohol are sometimes added to the first alcohol.

Eosin and Methylene-blue.—Harden in alcohol, Zenker's fluid, or corrosive sublimate; Zenker's fluid is especially recommended.

1. Stain paraffin sections in a 5 to 10 per cent. aqueous solution of eosin for five to twenty minutes or longer.
2. Wash in water to get rid of excess of eosin.
3. Stain in Unna's alkaline methylene-blue solution (see page 271), diluted 1–10 with water, for one-half to one hour, or use a stronger solution, and stain for a few minutes only.
4. Wash in water.
5. Differentiate and dehydrate in 95 per cent. alcohol followed by absolute alcohol until the pink color returns in the section.
6. Xylol.
7. Xylol balsam.

For celloidin sections use 95 per cent. alcohol, blot, and pour on xylol; repeat the last two steps until the specimen is clear.

It is important to get a deep stain with eosin, because the methylene-blue washes it out to a considerable extent. The eosin must be used first, because methylene-blue is readily soluble in an aqueous solution of eosin, and therefore is quickly extracted if the eosin is used after it, while on the other hand eosin is very slightly soluble in an aqueous solution of methylene-blue which is precipitated by any excess of eosin.

Diffuse or contrast-stains are useful to make prominent certain of the tissue-elements left uncolored by the nuclear stain. A greater richness of detail is obtained with diffuse stains if, after rather deep staining, the sections be washed out for some time in alcohol, because certain structures possess a greater affinity than others for certain diffuse stains, and by holding them are brought out sharply.

Of the diffuse stains, eosin, picric acid, and acid-fuchsin in Van Gieson's mixture are the ones most frequently employed.

Eosin is most frequently used as a contrast to alum-hematoxylin stains, but is often serviceable with alum-cochineal, methylene-blue, gentian-violet, etc. It brings out particularly well red blood-globules and smooth and striated muscle-fibers. The strength of the solutions used after hematoxylin varies from $\frac{1}{10}$ to $\frac{1}{2}$ per cent. according to the tissue and the fixative used. Zenker's preparations stain intensely in eosin, so that for them a very dilute solution is advisable. When desired as a contrast-stain to basic aniline dyes, eosin should be used first in a 5 or even 10 per cent. solution, because otherwise it is likely to be washed out by the nuclear stain.

Picric acid is used for contrast with the carmine stains, more rarely with alum-hematoxylin. Striated muscle-fibers and cornified epithelium are rendered especially prominent by it. To stain with picric acid it is only necessary to add a few drops of a saturated aqueous solution to a dish of water, or of a saturated alcoholic solution to a little alcohol, and allow sections to remain in the solution for a few seconds.

Van Gieson's stain (see p. 274), a mixture of picric acid and acid fuchsin, is excellent as a contrast-stain to alum-hematoxylin, especially when it is desirable to render prominent connective-tissue fibrillæ or certain pathological products. The nuclear stain with alum-hematoxylin must be rather deep, because the picric acid to some extent extracts or overpowers it.

1. Stain deeply in alum-hematoxylin.
2. Wash in water.
3. Stain in Van Gieson's solution three to five minutes.
4. Wash in water and dehydrate directly in
5. Alcohol, 95 per cent.
6. Oleum origani cretici.
7. Canada balsam.

Neutral Carmine (see page 269).—Neutral carmine is a diffuse stain, and is employed more especially for the central nervous system and for bone.

Filter one or two drops of the solution into 20 c.c. of distilled water, and leave the sections in the dilute solution over night. It is advisable to place a piece of filter-paper on the bottom of the dish for the sections to rest on, otherwise they may be stained on the upper side only. In double stains with hematoxylin and carmine the sections should be stained first in the hematoxylin and then thoroughly washed in water for six to twelve hours before they are stained in the carmine. After the carmine they are again to be thoroughly washed in water.

Combination Stains.—**Biondi-Heidenhain Stain** (see p. 275).—Tissues must be hardened in corrosive sublimate.

1. Stain paraffin sections six to twenty-four hours with the dilute solution.
2. Wash out a little in 90 per cent. alcohol.
3. Dehydrate in absolute alcohol.
4. Xylol.
5. Canada balsam.

It is important to place the sections directly from the staining fluid into the alcohol, because water washes out the methyl-green almost instantly.

Staining in Mass.—The staining of tissues in mass is a procedure much less employed in pathological than in normal histology, but still occasionally useful. For pathological tissues a variety of stains is generally necessary. It is therefore much better to make a series after one of the methods described, and then to stain the sections in whatever way seems best.

For staining in bulk only a limited number of solutions are available—either those, like alum-carmine and alum-cochineal, which do not stain beyond a certain point, or those, like lithium and borax-carmine and Heidenhain's hematoxylin, which may be decolorized so as to leave only the nuclei stained, The process of staining differs from that for sections only in the length of time required for each step. Tissues ½ cm. thick will need from one to two days in the staining solution.

MITOSIS.

For the study of karyomitosis it is important that the tissue be perfectly fresh—that is, just removed from a living animal or from one just dead—and that it be fixed in a suitable reagent as quickly as possible. The best results cannot be obtained with tissues put into a hardening fluid over half an hour after removal from a living animal. On the other hand, mitotic figures can be demonstrated in tissues which have been dead for some time (twenty-four hours or more) before being put into a fixing reagent, but the details of the figures are not so perfect as those in perfectly fresh tissues, and the figures are not so numerous, because some of them have completed their changes and can no longer be recognized. It is therefore evident that mitosis can be studied much better in tissues from the lower animals, or in tissues removed by operation from the human body, than in the organs and tissues removed at post-mortem examinations.

The choice of fixing reagents for the study of mitotic figures is important. The figures can often be demonstrated after hardening in alcohol or even in Müller's fluid, but for their careful study quicker and more perfect fixing reagents must be used. Nearly all of the reagents employed penetrate slowly, so that it is absolutely necessary for the best results that the tissue to be hardened be cut into very thin slices, rarely over 4 mm. in thickness and preferably not over 2 mm. The amount of fixing reagent used should always be at least ten to fifteen times as great as the volume of the tissue, and should be changed if it becomes cloudy.

The most important fixing reagents are—

1. Flemming's solution.
2. Hermann's solution.
3. Pianese's solution.
4. Zenker's fluid.
5. Corrosive sublimate.
6. Orth's fluid.

The first three solutions penetrate with much difficulty, so that tissues placed in them should be especially thin. The most generally useful stain for mitosis is probably safranin. The time of staining varies with the solution used. Babes' is the quickest. The mitotic figures should be stained deeply: then, when treated with alcohol slightly acidulated with hydrochloric acid, they will retain the color, while the resting nuclei will yield up most of theirs and become very pale or even colorless. In consequence of this intense stain mitotic figures can then be very readily found.

Directions for Staining Karyomitotic Figures with Safranin.—1. Stain paraffin sections five minutes to twenty-four hours, according to solution used.

2. Wash in water.

3. Wash in 95 per cent. alcohol to which are added a few drops of acid alcohol.

4. Wash in pure 95 per cent. alcohol, followed by absolute alcohol.

5. Xylol.

6. Xylol balsam.

For celloidin sections dehydrate in 95 per cent. alcohol, blot, and pour on xylol; repeat the last two steps until the specimen is clear. Safranin can be used after any of the above fixing reagents.

Other useful stains are carbol-fuchsin and aniline-gentian-violet, used in the same way as the safranin. The Gram-Weigert method gives good results after Flemming's solution.

After fixing in corrosive sublimate mitotic figures can be demonstrated by the Biondi-Heidenhain solution, which stains resting nuclei blue-violet and mitotic figures green. After Pianese's solution his special staining mixtures should be used (see page 275). His methods are said to give beautiful results.

SPECIAL STAINS FOR CERTAIN TISSUE-ELEMENTS OTHER THAN NUCLEI.

MASTZELLEN.

MASTZELLEN are found in the connective tissue, more especially in chronic inflammatory processes. Their protoplasmic granules stain intensely like bacteria with the basic aniline dyes. Several methods of staining the granules are given. With Unna's stains for plasma-cells a differential color-stain is obtained for the granules of the mastzellen.

Ehrlich's Method.—*A. General Stain.*—Harden in alcohol.

1. Stain with a saturated aqueous solution of dahlia.

2. Wash out with acidified water.

3. Dehydrate in alcohol; clear and mount in Canada balsam.

B. Specific Stain.—Only the protoplasmic granules are stained. Harden in alcohol.

1. Stain twelve hours in—

Absolute alcohol,	50 c.c.
Water,	100 "
Glacial acetic acid,	12.5 "
Dahlia,	q. s., so that the solution is almost saturated.

2. Wash out in alcohol, clear, and mount.

C. Ehrlich-Westphal Method.—Nuclei red; granules blue. Harden at least a week in alcohol.

1. Stain in the following solution twenty-four hours:

Alum-carmine solution,	200;
Saturated solution of dahlia in absolute alcohol,	200;
Glycerin,	100;
Glacial acetic acid,	20.

(Stir repeatedly, then allow the mixture to stand for some time.)

2. Decolorize for twenty-four hours in absolute alcohol.

3. Oil, Canada balsam.

Unna's Isolated Stains for Mastzellen.—Harden in alcohol. Nuclei blue; protoplasmic granules of "Mastzellen" red.

A.—1. Stain in polychrome methylene-blue solution, plus a little alum, for three hours to over-night.

2. Wash in water.

3. Absolute alcohol, oil of bergamot, balsam.

B.—1. Stain in polychrome methylene-blue solution one-quarter of an hour.

2. Wash in water.

3. Decolorize in glycerin-ether mixture for five to ten minutes.

4 Wash a long time in water.

5. Absolute alcohol, oil, balsam.

PLASMA-CELLS.

These are certain cells, much studied by Unna, which are very abundant in subacute and chronic pathological processes, and which are characterized by protoplasm which stains quite deeply in alkaline methylene-blue solutions. The two methods best suited for their demonstration furnish at the same time a differential color-stain for mastzellen. The granules of the latter are stained red, the plasma-cells are stained blue.

Unna's Differential Stains for Plasma-cells and Mastzellen.—Harden tissues in absolute alcohol.

A.—1. Stain paraffin sections in polychrome methylene-blue one-quarter of an hour to over-night.

2. Decolorize in a small dish of water, to which are added a few drops of glycerin-ether mixture.

3. Wash thoroughly in water.

4. Absolute alcohol, xylol, balsam.

B.—1. Stain in polychrome methylene-blue solution five to fifteen minutes.

2. Wash in water.

3. Decolorize and dehydrate in a ½ per cent. alcoholic solution of neutral orcein (about fifteen minutes).

4. Absolute alcohol, xylol, balsam.

CONNECTIVE-TISSUE FIBRILLÆ AND RETICULUM.

Several methods are available for the demonstration of connective fibrillæ and reticulum. The simplest is by means of Van Gieson's picric acid and acid-fuchsin solution, but it is applicable to the coarser fibers only. With Ribbert's method contrast stains are incompatible. The stain with aniline blue is believed to be better than any yet proposed, but is limited to tissues hardened in corrosive sublimate solution or in Zenker's fluid.

A. **Mallory's Aniline Blue Stain.**[1]—The following method is not absolutely differential because, besides connective-tissue fibrillæ and reticulum, it also stains certain hyaline substances, but these latter usually are so different morphologically that confusion cannot arise. The method is also useful for the study of fibrin, smooth and striated muscle-fibers, and amyloid.

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[1] Mallory: *The Journal of Experimental Medicine*, 1900, v., 15.

1. Fix in corrosive sublimate or in Zenker's fluid.
2. Imbed in celloidin or paraffin.
3. Stain sections in a $\frac{1}{20}$ to $\frac{1}{10}$ of a 1 per cent. aqueous solution of acid fuchsin one to three minutes.
4. Wash in water.
5. Place in a 1 per cent. aqueous solution of phospho-molybdic acid for one minute or longer (use glass or platinum).
6. Wash in two changes of water.
7. Stain in the following solution for five to twenty minutes or longer:

Aniline blue soluble in water (Grübler),	0.5;
Orange G (Grübler),	2.0;
Oxalic acid,	2.0;
Water,	100.0.

8. Wash in water.
9. Dehydrate in 95 per cent. alcohol.
10. Blot on the slide, and clear in xylol, or clear in oleum origani cretici.
11. Xylol balsam.

The fibrillæ and reticulum of connective tissue, amyloid, mucus, and certain other hyaline substances stain blue; nuclei, protoplasm, elastic fibers, axis-cylinders, neuroglia-fibers, and fibrin red; red blood-globules and myelin-sheaths yellow. The various structures do not stain with equal intensity, so that certain ones are brought out with great sharpness. This is particularly true of the fibrillæ and reticulum of connective tissue, and of fibrin and smooth and striated muscle-fibers.

If it is desired to bring out the connective tissue as sharply as possible, omit the staining with acid fuchsin. Then the nuclei and protoplasm stain yellow, and the blue fibrillæ and reticulum stand out more prominently.

B. **Van Gieson's Picro-fuchsin Stain.**—The proportions given are those recommended by Freeborn. Occasionally it will be found necessary to increase the proportion of the acid fuchsin.

1. Harden in chrome salts or in corrosive sublimate. The results after alcohol are not so good.

2. Stain deeply in alum-hematoxylin. 12 hrs

3. Wash in water.

4. Stain for three to five minutes in Van Gieson

1 per cent. aqueous solution of acid fuchsin,	5 c.c.
Saturated aqueous solution of picric acid,	100 "

ash in H_2O dann a

5. Dehydrate in 95 per cent. alcohol. directly

6. Oleum origani cretici.

7. Canada balsam.

C. **Ribbert's Phosphomolybdic-acid Hematoxylin Stain.**—1. Fixation in alcohol is preferable.

2. Place sections in a 10 per cent. solution of phosphomolybdic acid for five to thirty seconds (use glass or platinum needles).

3. Wash quickly in water.

4. Stain five minutes or less in phosphomolybdic acid hematoxylin. (For this purpose the solution can be used after it is twenty-four hours' old.)

5. Water.

6. Alcohol, oil, Canada balsam.

Fibrillæ deep blue; other tissue-elements grayish-green; contrast-stains incompatible. The method is recommended for staining the finest fibers of connective tissue.

D. **Unna's Orcein Stain.**—1. Harden in alcohol.

2. Stain in the concentrated solution of polychrome methylene-blue five minutes.

3. Wash in water.

4. Decolorize, differentiate, and stain in a 1 per cent. solution of orcein in absolute alcohol fifteen minutes.

5. Wash in absolute alcohol.

6. Oil of bergamot.

7. Balsam.

Nuclei, dark blue; protoplasm, pale blue; elastic and connective-tissue fibers, deep orcein red; smooth muscle-fibers, bluish; mastzellen granules, red; protoplasm of plasma-cells, deep blue.

E. **Mall's Differential Method for Reticulum.**[1]—1. Digest frozen sections of fresh tissue, 40 to 80μ thick, for twenty-four hours in the following solution:

Parke, Davis & Co.'s pancreatin,	5 grams;
Bicarbonate of sodium,	10 "
Water,	100 c.c.

2. Wash carefully in clean water.
3. Place sections in a test-tube half full of water, and shake thoroughly in order to remove all of the cellular débris.
4. Spread out on slide, and allow to dry.
5. Allow a few drops of the following solution to dry on surface:

Picric acid,	10 grams;
Absolute alcohol,	33 c.c.;
Water,	300 "

6. Stain for about half an hour in the following solution:

Acid fuchsin,	10 grams;
Absolute alcohol,	33 c.c.;
Water,	66 "

7. Wash in the picric acid solution for a moment.
8. Alcohol, xylol, balsam.

ELASTIC FIBERS.

Elastic fibers are not affected by dilute caustic soda or potash, or by acids. These reagents are often used, therefore, to demonstrate elastic fibers in the fresh condition, as, for example, in sputum, because they render them prominent by clearing or destroying the other tissues. The fibers show a marked affinity for osmic acid, staining with greater rapidity than most other tissue-elements.

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[1] Mall: *Johns Hopkins Hospital Reports*, 1896, i., 171.

For bringing out elastic fibers in sections of hardened tissues there are two excellent differential stains, of which the first, Weigert's, is particularly recommended for the simplicity of the method and the sharpness and intensity of the pictures it gives.

A. **Weigert's Stain for Elastic Fibers.**[1]—Fixation in alcohol or formaldehyde preferable, but other fixing reagents give good results. Imbed in celloidin or paraffin.

1. Stain sections twenty minutes to one hour in the following solution:

Fuchsin,	2;
Resorcin,	4;
Water,	200.

Boil the solution in a porcelain dish; when briskly boiling add 25 c.c. of liquor ferri sesquichlorati; stir and boil for two to five minutes. A precipitate forms. Cool and filter. The filtrate is thrown away. The precipitate remains on the filter-paper until all the water has drained away or until the precipitate has thoroughly dried. Then return filter and precipitate to the porcelain dish, which should be dry, but which should contain whatever part of the precipitate remained sticking to it. Add 200 c.c. of 95 per cent. alcohol, and boil. Stir constantly, and fish out the filter-paper as the precipitate is dissolved off. Cool; filter; add alcohol to make up the 200 c.c. Add 4 c.c. of hydrochloric acid.

2. Wash off in alcohol.

3. Blot with filter-paper, and add xylol quickly; repeat the blotting, followed by xylol, two or three times until the section is perfectly cleared.

4. Xylol balsam.

Sections can be stained for several hours. If the rest of the tissue is over-stained, differentiate in acid alcohol; if the sections are too deeply stained, the color cannot be washed out. Diffuse staining can be avoided by diluting the stain. The elastic fibers appear dark blue, almost black, on a clear background. The nuclei can be stained red with carmine before or after the staining of the fibers.

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[1] Weigert: *Centralblatt für allg. Pathologie*, 1898, ix., 289.

The solution keeps for months.

If it be desired to keep sections for some time before mounting, wash them in alcohol, and place in water.

B. **Unna's Orcein Method for Elastic Fibers.**—Unna's latest method of using orcein is as follows, and can be highly recommended:

1. Stain sections in the following solution:

Orcein (Grübler),	1;
Hydrochloric acid,	1;
Absolute alcohol,	100.

Place the sections in a dish and pour over them enough of the solution to cover them. Warm gently in an incubator or over a small flame for ten to fifteen minutes until the solution thickens, or leave in the solution at room-temperature over night.

2. Wash off thoroughly in dilute alcohol (70 per cent.).
3. Wash in water to get rid of all the acid and to fix the color.
4. Alcohol.
5. Oil.
6. Balsam.

The washing in water is not absolutely essential.

Elastic fibers are stained of a deep silky-brown color, connective tissue a pale brown. If it is desirable to have only the elastic fibers stained, wash for a few seconds in 1 per cent. hydrochloric-acid alcohol before washing in water. The nuclei can be brought out by staining in Unna's polychrome methylene-blue solution after washing the sections in water.

MUSCLE-FIBERS.

Smooth and striated muscle-fibers are brought out fairly sharply in contrast with other tissue-elements by means of eosin and of Van Gieson's picro-fuchsin stain. They are brought out much more clearly, however, by the aniline blue stain recommended for connective tissue. When it is desired to render the muscle-fibers as prominent as possible, use a little stronger solution of acid fuchsin, and wash out longer in the alcohol. The muscle-fibers appear red, the connective-tissue fibrillæ and reticulum blue.

THE CENTRAL NERVOUS SYSTEM.

In the preservation of the central nervous system the special structures which require consideration are the ganglion-cells, including both the dendritic and the axis-cylinder processes, the myelin sheaths, and the neuroglia-fibers. No one fixing reagent is suited for the best preservation of all of them, unless possibly it be formaldehyde.

The main fixing fluids for the nervous system until within a very short time have been various solutions of the chrome salts, particularly of bichromate of potassium, either alone or in combination with sulphate of sodium, as in the well-known Müller's fluid. The chief objections to the chrome salts as fixatives are that they penetrate and harden very slowly, and do not preserve properly either the ganglion-cells or the neuroglia-fibers. On the other hand, they probably preserve the axis-cylinders as well as any reagent we yet know, and are invaluable for their property of entering into some chemical combination with myelin, in consequence of which it is possible to obtain by the method originated by Weigert a differential stain of the myelin sheaths.

The new fixing reagent, formaldehyde, seems likely to find its greatest use histologically as a fixative of the central nervous system. It penetrates and hardens up to a certain degree with great rapidity. It also preserves in certain structures the special chemical properties on which certain differential stains depend. Small pieces of nervous tissue are properly fixed in the standard solution (4 per cent. solution of formaldehyde gas) in four days. A whole brain will be so hardened in ten days to two weeks that thin serial sections can be made through it without fear of the slices altering their shape in the least. The process could undoubtedly be hastened by injecting the arteries.

It must be borne in mind, however, that for most purposes formaldehyde must be followed by other reagents before the structures and their chemical properties preserved by it are properly fixed so that they will not be altered when transferred to alcohol. In other words, formaldehyde may be looked upon as a very quick preliminary fixing reagent. The hardening of brains entire in it is not recommended, except in certain cases—for instance, of cysts, hemorrhages, or occasionally of tumors—where the gross lesions and the tracts or structures affected by them are of more importance than the finer histological changes. For the proper preservation of ganglion-cells and of neuroglia-fibers very small pieces must be taken and fixed by the special methods given; but if the main object is to trace system-degenerations, much larger pieces, or even the whole brain, may be taken, because the myelin-sheaths change comparativly very slowly after death.

The stains for the central nervous system may be divided into two classes, general and differential. For nearly all of them preliminary fixation in formaldehyde is advisable or possible. This renders the immediate preservation of nervous tissue very simple, and at the same time allows a variety of mordanting and staining methods to be used later.

The staining of the various histological elements of the central nervous system and the fixing reagents best suited for each of them will be considered under the following headings:

- General Stains.
- Stains for ganglion-cells,
  - Protoplasmic granules;
  - Dendritic and axis-cylinder processes;
  - Axis-cylinders and their terminal processes.
- Stains for the myelin-sheath.
- Stains for the neuroglia-fibers.

General Stains.—General stains include the ordinary nuclear stains, with or without a contrast-stain, and certain diffuse single or combined stains which color the nuclei, the cell-protoplasm, including to a varying extent the dendritic processes of the ganglion-cells, the axis-cylinders, and the neuroglia-fibers. The different stains vary somewhat in regard to the structures which they bring out most prominently.

The best fixation for the general stains is, on the whole, that used for mordanting the myelin-sheaths; in other words, formaldehyde combined with or followed by a chrome salt (see page 322). Occasionally the fixatives employed for other tissues are indicated. Alum-hematoxylin, followed by eosin, is always a useful stain. The eosin if deep enough brings out fairly well both the dendrites and the axis-cylinders. The basic aniline dyes, especially methylene-blue, find their greatest use in Nissl's method of staining the protoplasmic granules of the ganglion-cells.

The various carmine solutions, particularly neutral, ammonia, and picro-carmine, have long been the favorite diffuse stains for the central nervous system, but the uncertainty of their action and the difficulty of always getting a good staining solution has gradually led to the introduction of more reliable methods. Of these the simplest, quickest, and in many ways the most generally useful is

A. **Van Gieson's Picro-fuchsin Stain.**—Although this mixture of acid fuchsin and picric acid may be made up in the way originally recommended, the following exact proportions, given by Freeborn for staining nervous tissues, will be found generally preferable:

1 per cent. aqueous solution of acid fuchsin,	15 c.c.;
Saturated aqueous solution of picric acid,	50 "
Water,	50 "

1. Stain sections first rather deeply in alum-hematoxylin.
2. Wash in water.
3. Stain in above solution three to five minutes.
4. Dehydrate in alcohol.
5. Oil, Canada balsam.

The nuclei appear bluish red, the ganglion-cells and processes red, the axis-cylinders brownish-red, the myelin-sheaths yellow, the neuroglia-fibers orange red, connective-tissue fibrillæ deep red. After certain reagents this solution will not give a sufficiently intense stain. In such cases a mixture of 1 part of a 1 per cent. solution of acid fuchsin to 2 parts of a saturated solution of picric acid is recommended.

B. **Phosphotungstic-acid hematoxylin** (see page 268) will be found of much value as a general stain for the central nervous system if employed in the manner recommended for neuroglia-fibers after fixation by the method there given, because a greater differentiation of the various tissue-elements is obtained than by any other method. Preliminary staining in Van Gieson's mixture is often an advantage.

C. **Phosphomolybdic-acid Hematoxylin** (see page 267). —1. Stain sections twenty minutes to one hour.

2. Wash out in two or three changes of 50 per cent. alcohol until the celloidin becomes completely decolorized (about five minutes).

3. Dehydrate in 95 per cent. alcohol.

4. Oil, Canada balsam.

The ganglion-cells are often over-stained, especially if the tissue has been hardened but recently. The method is particularly good for bringing out the axis-cylinders and the neuroglia-fibers.

D. **Aniline Blue Stain** (see page 269).—The method recommended for connective-tissue fibers will also be found very useful for the study of the nervous system. The best results are obtained after fixation in Zenker's fluid, but fair results can also be obtained after fixation by the method advised for neuroglia-fibers—namely, formaldehyde, picric acid, and bichromate of ammonium, provided the following changes are made in the method: Use a 1 per cent. solution of acid fuchsin; mordant for several minutes in the phosphomolybdic acid; do not stain more than one to three minutes in the aniline blue solution.

E. **Nigrosin.**—1. Stain sections in a concentrated aqueous solution of nigrosin five to ten minutes.

2. Decolorize and dehydrate in weak, then in strong, alcohol.

3. Oil, Canada balsam.

The stain is not very sharp, but is simple and useful, particularly for low-power observation.

Stains for Ganglion-cells; Protoplasmic Granules. — *A.* **Nissl's Latest Improved Method.** — 1. Carefully harden pieces of tissue not over 1 to 1.2 cm. in diameter in 96 per cent. alcohol.

2. Cut sections without imbedding, as follows: Remove excess of alcohol from tissue with filter-paper; dip base of specimen in thick celloidin; mount on block; harden in 96 per cent. alcohol. Moisten knife with 96 per cent. alcohol. Sections should always be under $\frac{1}{100}$ mm. in thickness. Preserve sections in 96 per cent. alcohol.

3. Stain the sections in the following solution heated over a flame until it bubbles noisily (60°–70° C.):

Methylene-blue, B patent,	3.75;
Venetian soap,	1.75;
Distilled water,	1000.

4. Wash out in—

Aniline oil,	10 parts;
96 per cent. alcohol,	90 "

until the color is no longer given off in coarse clouds.

5. Transfer section to slide; dry with filter-paper and cover with oil of cajuput.

6. Blot with filter-paper, and then wash with a few drops of benzine.

7. Add a few drops of benzine-colophonium (made by dissolving colophonium in benzine for twenty-four hours and then decanting).

8. Pass slide through flame to drive off the benzine, which will inflame, but if blown out immediately the specimen will not be injured. Repeat the process until all the benzine is driven off.

9. Cover-slip. Warm the slide, so that the colophonium will spread out evenly between the cover-slip and the slide.

The specimen is now mounted in a medium in which diffusion of color cannot take place, so that the stain is practically permanent. The best results are obtained with tissues which have not been hardened in alcohol over one to four days. Contact with water, weak alcohol, and ether must be avoided.

B. **Lenhossek's Method for Ganglion-cells.**—The following method will be found simpler, but the specimens are not permanent:

1. Harden sections in 90 per cent. alcohol, then in 96 per cent., or in formaldehyde followed by alcohol. Do not keep the tissues too long in alcohol.
2. Imbed sections in celloidin or paraffin, or cut without imbedding, as in Nissl's method.
3. Stain sections in a completely saturated solution of thionin for five minutes.
4. Wash for a few seconds in water.
5. Differentiate in aniline, 1 part;
   Absolute alcohol, 9 parts.
   Do not decolorize too long.
6. Clear in oleum cajuputi.
7. Xylol.
8. Canada balsam.

The granulations can be shown by other stains, such as safranin, fuchsin, dahlia, alum-hematoxylin.

Ganglion-cells; Dendritic and Axis-cylinder Processes.—**Golgi's Methods.**—Golgi's methods, although of the greatest value in the study of the normal histology of the central nervous system, are of very little use in the study of its pathology. The reason of this is the very peculiarity that makes the method of value in normal histology—namely, that it picks out here and there a cell and stains it with all its wealth of processes more or less completely, while the neighboring cells are left colorless. If all of the cells and their processes were stained, the picture presented would be a confused mass. In pathological histology, where the presence or absence of certain cells or processes is of paramount importance, it is of primary necessity that every cell within a given area shall be perfectly stained.

Golgi introduced three different methods of obtaining the stain now called after his name. They are spoken of as the slow, the mixed, and the short methods. Golgi himself employed principally the first two methods, and they are still used for the study of the developed brain and cord.

The quick method exclusively has been used by Ramon y Cajal and other recent investigators for the study of embryonic nervous tissue.

The following points are to be borne in mind: The tissue should be as fresh as possible, and should be cut into small pieces, not over 1 to 1½ cm. thick—for the quick method even thinner. With the corrosive-sublimate method, however, larger pieces can be used.

Large quantities of the solutions should be used—at least ten times the volume of the specimen It is best to keep the specimens in the solution in the dark, especially in using the corrosive-sublimate method.

Golgi's Slow Method.—1. Harden the tissues in a 2 per cent. solution of bichromate of potassium two to six weeks. In summer fifteen to twenty days are sufficient; in winter, unless the temperature is kept at 25° C., one to one and a half months will be required. Keep the specimens in the dark. Large amounts of the solution should be used, and it should be frequently changed, especially during the first week.

2. Transfer either to (*a*) a ¾ per cent. solution of nitrate of silver for twenty-four to forty-eight hours; a longer time will do no harm; or to (*b*) a ½ per cent. solution of corrosive sublimate—small pieces eight to ten days, large pieces two months or more. Change the solution frequently during the first few days; later only when the solution gets yellow.

This second procedure is recommended for larger pieces of tissue than can properly be impregnated by (*a*).

Golgi's Mixed Method.—1. Harden small pieces of tissue for three to five days or longer in a 2 per cent. solution of bichromate of potassium at 25° C., in the dark.

2. Transfix to a mixture of—

1 per cent. solution of osmic acid,	2 parts;
2 per cent. solution of bichromate of potassium,	8 "

for three to eight days.

3. Place in a $\frac{3}{4}$ per cent. solution of nitrate of silver for twenty-four to forty-eight hours.

Golgi's Quick Method.—1. Small pieces of fresh tissue are placed directly in the following solution:

1 per cent. solution of osmic acid,	1 part;
3.5 per cent. solution of bichromate of potassium,	4 parts,

for several days (three to eight).

2. They are then transferred to a large amount of a $\frac{3}{4}$ per cent. solution of nitrate of silver for one, two, or six days.

The length of time the tissues should remain in the osmic-acid and bichromate-of-potassium solution depends on what elements it is desired to impregnate. In the human cord the time is in general the following:

1. Neuroglia,	2–3 days:
2. Nerve-cells,	3–5 "
3. Nerve-fibers and collaterals,	5–7 "

The further treatment of the tissues impregnated by these methods is as follows: Alcohol must be avoided as much as possible. The tissues are usually firm enough to cut after the impregnation; if not, place in absolute alcohol for fifteen to thirty minutes. The sections should be rather thick, $\frac{1}{20}$ to $\frac{1}{10}$ mm. They may be made free hand with a razor or in the microtome. For either method the tissues can be held between pieces of elder-pith, or may be quickly imbedded in celloidin by dehydrating for a few minutes in absolute alcohol and then placing in a thick solution for five minutes. From the celloidin they are mounted in elder-pith or on blocks, and placed for a short time in 80 per cent. alcohol to harden.

Treatment of Sections.—1. Dehydrate quickly in alcohol.

2. Clear in oil of cloves or bergamot.

3. Wash off with xylol.

4. Mount without a cover-glass in xylol damar, and dry quickly at 40° C.

The mounted sections must be protected from the light and from dust as much as possible. Cajal has modified Golgi's quick method by repeating the steps (Cajal's so-called double method) so as to get a more perfect impregnation. The same osmic-acid and bichromate-of-potassium solution may be used over again, or a fresh solution, containing about one-half as much osmic acid, is made up fresh. The silver solution should be taken fresh each time. Lenhossek, Weigert, and others have obtained very good Golgi preparations with tissues first fixed in formaldehyde.

Of the various methods proposed for fixing the Golgi stains so that contrast-stains could be used with them and the specimens protected by cover-slips, the simplest and most practical seems to be that advocated by Kallius.

The Method of Kallius for Fixing Golgi Stains.—The method depends on the employment of a photographic developer to reduce the bichromate of silver to metallic silver.

1. Place sections for several minutes in a solution composed of 1 part of the following developer:

Hydrochinon,	1;
Sulphite of sodium,	8;
Carbonate of potassium,	1.5;
Water,	575,

plus one-third to one-half as much absolute alcohol until the sections become gray to black in color. If too much alcohol is added, the carbonate of potassium will be precipitated, but will redissolve on the addition of a little more developer.

2. 70 per cent. alcohol for ten to fifteen minutes.

3. Hyposulphite of sodium (20 per cent. aqueous solution).

4. Wash thoroughly in a large amount of water for twenty-four hours.

5. Alcohol, oil, Canada balsam; cover-glass.

Cox's Modification of Golgi's Corrosive-sublimate Method.—The same black pictures are obtained by this method as by Golgi's, but with this difference, that nearly all of the cells in the section are impregnated. This is an advantage when the topographical arrangement of the cell-layers is desired, but a disadvantage when it comes to the study of individual cells, because on account of the luxuriance of the impregnation such a study is rendered impossible. Small pieces of nervous tissue are placed in the following solution:

Bichromate-of-potassium 5 per cent. solution,	20;
Corrosive-sublimate 5 per cent. solution,	20;
Distilled water,	30–40;
Simple chromate-of-potassium 5 per cent. solution,	16.

The time required for impregnation is a month in summer and two to three months in winter. The after-treatment is the same as for Golgi preparations.

Axis-cylinders and their Terminal Processes.—The three methods most in use for the study of central and peripheral nerve-fibers and their terminations are the gold, the Golgi, and the methylene-blue methods. All three may give beautiful results, but, as a rule, they are very unreliable. Their use is confined almost wholly to the study of normal tissues.

Gold Stain for Nerve-fibers.—For the application of the gold method to fresh tissues see p. 285.

Various attempts have been made to devise a reliable method of employing chlorid of gold for staining nerve-fibers in sections of hardened tissues. The results have not been altogether successful. The best results can probably be obtained by—

A. Gerlach's Method.—1. Harden tissues in a 1–2 per cent. solution of bichromate of ammonium for one to three weeks; cut sections without passing through alcohol, which must be avoided.

2. Place the sections in a very dilute solution ($\frac{1}{100}$ per

cent.) of the double chlorid of gold and potassium very slightly acidulated with hydrochloric acid, for ten to twelve hours, until they become slightly violet in color.

3. Wash in a solution of hydrochloric acid 1, to water 2000–3000.

4. Place for ten minutes in a $\frac{1}{10}$ per cent. solution of hydrochloric acid in 60 per cent. alcohol.

5. Absolute alcohol, oil of cloves, Canada balsam.

Another method frequently recommended is the following:

B. Freud's Gold Stain for Nerve-fibers.—1. Harden tissues in Erlicki's or Müller's fluid, followed by alcohol. Imbed in celloidin.

2. Stain sections three to five hours in 1 per cent. solution of chlorid of gold, and 95 per cent. alcohol, equal parts.

3. Wash in water.

4. Reduce in—

Caustic soda,	1;
Distilled water,	6,

for two to three minutes.

5. Wash in water.

6. Place for five to fifteen minutes in a 10 per cent. solution of iodid of potassium.

7. Wash in water.

8. Alcohol, oil, Canada balsam.

C. Stroebe's Aniline-blue Stain for Nerve-fibers in Hardened Sections.—Harden tissues in Müller's fluid. 1. Stain one-half to one hour in a saturated aqueous solution of aniline-blue.

2. Wash in water.

3. Transfer to a small dish of alcohol to which are added 20 to 30 drops of a 1 per cent. alcoholic solution of caustic potash (caustic potash 1 to alcohol 100: let the mixture stand for twenty-four hours; then filter). In one to several minutes the sections become bright brownish-red and transparent.

4. Transfer to distilled water for five minutes. The section becomes bright blue again.

5. Stain in a half-saturated aqueous solution of safranin one-quarter to one-half hour long.

6. Wash out and dehydrate in absolute alcohol.

7. Xylol, Canada balsam.

D. Chlorid-of-iron and dinitroresorcin method for the study of degenerated peripheral nerves:

1. Place fresh pieces of peripheral nerves for several days in a solution of—

Chlorid of iron,	1 part;
Distilled water,	4 parts.

2. Wash out thoroughly in water.

3. Transfer to a saturated solution of dinitroresorcin in 75 per cent. alcohol for several weeks.

4. Wash, dehydrate, imbed, etc.

A permanent green color is formed which stains the nerves green and brings out the green axis-cylinders very sharply.

The stain will succeed with preparations which have been hardened in Flemming's solution or Müller's fluid.

Golgi's methods are sometimes employed for the study of the terminal processes of nerve-fibers (for directions see p. 314).

Methylene-blue Stain for Nerve-fibers.—The methylene-blue method is due to Ehrlich. Many modifications of the original procedure have been suggested with a view to making the results surer or the specimens more permanent. Tissues can be stained either by injection or by immersion.

The methylene-blue used should be Grübler's "rectified methylene-blue for vital injection."

For injection in the blood- or lymph-vessels of live or dead animals a 1 to 4 per cent. solution in normal salt solution is recommended. The injected organs are exposed to the air until a bluish tint is visible. As soon as the greatest intensity of stain is reached (five minutes to two hours) the color in the preparation is fixed by placing small bits of the tissue in a freshly-filtered, cold, saturated, aqueous solution of picrate of ammonium, or, better still, in the solution given below, recommended by Bethe.

Very small or thin pieces of tissue intended for staining by immersion (the method employed for human tissues) are placed in a very dilute solution ($\frac{1}{16}$–$\frac{1}{15}$ per cent.) of methylene-blue in normal salt solution. Lavdowski recommends very highly a solution of methylene-blue in egg-albumin, either alone or combined with chlorid of sodium or ammonium. The white of egg is freed from the thicker portions or filtered. When the experiment is to last some time, add to the egg-albumin an equal part of a $\frac{1}{2}$ per cent. solution of chlorid of sodium or of a $\frac{1}{4}$ per cent. solution of chlorid of ammonium. The tissue, protected by a large dish, is exposed to the air for fifteen minutes to twelve hours, until the maximum stain is obtained.

The stain may then be fixed by the method already given, or, better still, in the following manner:

Bethe's Method of Fixing Methylene-blue Stains of Nerve-fibers.—1. Wash off excess of color with normal salt solution.

2. Place in—

Molybdate of ammonium,	1 gr.;
Distilled water,	10 c.c.;
Peroxid of hydrogen,	1 "
Hydrochloric acid,	1 drop.

A precipitate forms on making up the solution, but disappears on shaking. The solution will keep eight days, but is best made up fresh each time. It should be used as cold as possible, preferably surrounded by a mixture of ice and salt. Leave the tissue in the cold solution for from two to five hours, and then for a while longer at the room-temperature.

3. Wash one half to two hours in running water.

4. Dehydrate and harden as quickly as possible (not over twelve to twenty-four hours) in cold absolute alcohol. (The color is soluble in warm alcohol.)

5. Clear in xylol.

6. Imbed in paraffin.

The sections may be mounted directly or brought into water and stained with alum-cochineal for contrast. If a little osmic acid be added to the fixing solution after the specimens have been in it for a while, a more permanent methylene-blue stain is obtained.

Stains for the Myelin-sheath.—The myelin-sheath of nerve-fibers is a form of fat, and like it possesses the property of reducing osmic acid, by means of which a selective sheath stain can be obtained. Unfortunately, however, the osmic acid penetrates to but a very slight depth.

The differential hematoxylin stain, originated by Weigert, and ordinarily used, depends on some chemical reaction which takes place between the myelin and a chrome salt, in consequence of which the myelin is fixed so that it will not later be dissolved out by alcohol or ether, and at the same time is so mordanted that it can be deeply stained with hematoxylin, to which it clings when treated with certain decolorizers. This reaction between the myelin and the chrome salt takes place very slowly at the ordinary temperature; six weeks to several months are usually required. Weigert has lately published a method depending on the interaction of two chrome salts in the same solution, in consequence of which the time needed for this reaction or mordanting is reduced to four days. The solution may be used alone, but is best combined with formaldehyde or used after it. Large masses of nervous tissue, like the medulla and pons or the basal ganglia, should be hardened in formaldehyde for one to three weeks, and then cut into parallel slices not over 1 cm. thick for mordanting by Weigert's quick method.

1. **To Mordant the Myelin-sheaths.**—*A. The Older Method.*—1. Harden in Müller's fluid six to twelve weeks.

2. Transfer directly to 80 per cent. alcohol.

B. A More Recent Method, Much to Be Preferred to A.—1. Fix in a 4 per cent. solution of formaldehyde (*i. e.*, 10 parts of the saturated 40 per cent. solution to 90 parts of water) for one to three weeks or longer.

2. Harden in Müller's fluid for six to twelve weeks, or in a 5 per cent. solution of bichromate of ammonium for four

to six weeks. If the tissues are placed in a thermostat at 37° C., only one to two weeks are required.

3. Transfer to 80 per cent. alcohol.

2. **Weigert's Quick Method of Mordanting Myelin-sheaths.**—1. Fix the tissues in a 4 per cent. solution of formaldehyde (10 per cent. solution of formaline) for four days to several weeks. Four days are sufficient for pieces not over 1 cm. thick. The solution, of which several times the volume of the specimen must be taken, is changed at the end of twenty-four hours. The tissue may remain indefinitely in it.

2. Mordant the myelin-sheaths in the following solution;

Bichromate of potassium,	5;
Chrome alum,	2;
Water,	100,

for four days. The tissues should not exceed 1 cm. in thickness, and should rarely be left in the solution longer than four days, because they will become brittle.

3. Transfer directly to alcohol (80 per cent.), and keep in the dark until wanted for imbedding, changing the alcohol occasionally as it becomes colored.

Steps 1 and 2 may be combined by adding 4 per cent. of formaldehyde to the mordanting solution and placing fresh tissues directly in the mixture.

Weigert's quick mordant can be highly recommended, both on account of the short time it requires and because the stains obtained after it are very satisfactory. The greatest objection to it is that exposure for over three to four days to the mordant renders the cerebrum, and, to a less extent, the cord, very brittle.

A. **Weigert's Myelin-sheath Stain.**—1. Fix and mordant the tissues in one of the ways already described.

2. Dehydrate in alcohol without washing out in water.

3. Imbed in celloidin.

4. Place sections for twenty-four hours in the following solution, which Weigert now recommends in place of the simple saturated solution of acetate of copper formerly advised:

Acetate of copper,	5. ;
Acetic acid, 36 per cent. solution,	5. ;
Chrome alum,	2.5 ;
Water,	ad 100.

(For method of preparation see page 329).

5. Stain fifteen minutes to twenty-four hours in—

Hematoxylin,	1 ;
Absolute alcohol,	10 ;
Saturated aqueous solution of carbonate of lithium,	1 ;
Water,	90.

Keep a 10 per cent. solution of hematoxylin in alcohol on hand, so that it will be ripe. Combine with the carbonate of lithium and the water at the time of using.

6. Wash thoroughly in water.

7. Decolorize in—

Borax,	4 ;
Ferricyanide of potassium,	5 ;
Water,	200,

until the gray substance is distinctly yellow.

8. Wash thoroughly in water.

9. Dehydrate in 95 per cent. alcohol.

10. Clear in aniline oil 2, xylol 1.

11. Canada balsam.

Weigert has given up his direct myelin-sheath stain because the preparations do not keep.

B. **Pal's Modification of Weigert's Myelin-sheath Stain.**—1. Fixation as for Weigert's method.

2. Place sections for several hours in a ½ per cent. aqueous solution of chromic acid, or for a longer time in a 2–3 per cent. solution of bichromate of potassium. This step is often omitted, especially when the tissues have been but recently mordanted.

3. Transfer sections to Weigert's hematoxylin solution for twenty-four to forty-eight hours (if necessary for an hour in the incubator at 37° C.).

4. Wash in water plus 1 to 3 per cent. of a saturated aqueous solution of carbonate of lithium until the sections appear of a uniform deep-blue color.

5. Differentiate for twenty seconds to five minutes in a ¼ per cent. aqueous solution of permanganate of potassium until the gray matter looks brownish-yellow.

6. Transfer to the following solution:

Oxalic acid,	1;
Sulphite of potassium,	1;
Water,	200,

for a few seconds until the gray matter is colorless or nearly so.

7. Wash thoroughly in water.

8. Dehydrate in 95 per cent. alcohol.

9. Oil, Canada balsam.

Steps 5 and 6 sometimes have to be repeated when the differentiation has not been complete.

Of all the numerous modifications of Weigert's original myelin-sheath stain, the only one that has found general acceptance is Pal's. It has the following advantages: It gives very clear pictures; everything except the sheaths is completely decolorized, so that contrast-stains are possible; it is more successful with thick sections than Weigert's method; the separate steps are quicker. On the other hand, the danger of decolorizing the sheaths of the finer fibers is greater.

C. **Exner's Method of Demonstrating Myelin-sheaths.**—The tissue should be obtained as soon as possible after death, although the method will succeed with tissues even over twelve hours old.

1. Place pieces of brain or cord not over ½ cm. thick in a 1 per cent. aqueous solution of osmic acid, using at least ten times as much fluid as the volume of the specimen.

2. Change the osmic-acid solution on the second day.

3. After five or six days wash thoroughly in water.

4. Dehydrate, imbed, etc.

5. Examine sections in glycerin rendered slightly ammoniacal.

The myelin-sheaths appear gray to black. The preparations are not permanent.

This procedure has been almost entirely replaced by Weigert's method, which has numerous advantages. Lately, however, it has been brought forward again by Heller, who uses a photographic developer to reduce the osmic acid and to make possible permanent mounts. He has lately published the following method for sections, but it cannot be unconditionally recommended:

D. **Heller's Method for Staining Myelin-sheaths with Osmic Acid.**—1. Harden as for the Weigert method (Heller used Müller's fluid).

2. Imbed in celloidin.

3. Place sections in a 1 per cent. aqueous solution of osmic acid for ten minutes in thermostat or for half an hour at room-temperature.

4. Wash in water.

5. Reduce in the following developer:

Sulphate of sodium,	125;
Carbonate of sodium,	70;
Water,	500;
Pyrogallic acid,	15.

6. Wash in water.

7. Differentiate in an aqueous solution of permanganate of potassium, ¼ per cent. or less.

8. Remove the brown of the permanganate of potassium in a 1 per cent. aqueous solution of oxalic acid.

9. Wash in water.

10. Alcohol, oil, Canada balsam.

By a modification of Heller's method Robertson claims to get better results.

1. Harden in Weigert's chrome-alum-copper solution plus 4 per cent. of formaldehyde; in other words, use the mordant for neuroglia-fibers (page 329) eight to ten days.

2. Wash off in water.

3. Alcohol; imbed in celloidin.

4. Stain sections in a 1 per cent. solution of osmic acid half an hour in the dark.

5. Place in a 5 per cent. aqueous solution of pyrogallic acid for half an hour.

6. Differentiate in a $\frac{1}{4}$ per cent. aqueous solution of permanganate of potassium one to four minutes.

7. Remove brown color in 1 per cent. oxalic acid three to five minutes.

8. Alcohol, oil, balsam.

It is important to wash carefully in water between each of the staining steps.

Stains for Neuroglia-fibers.—It is possible to obtain a differential stain of the neuroglia-fibers in man by three different methods, provided the first two of the following steps are complied with:

1. The tissue must be as fresh as possible. The best results are obtained with tissues placed in the fixing solution within one hour after death. After four to six hours the results are only fair; after twenty-four hours they are practically *nil.* The chemical property in the neuroglia-fibers on which the differential stain depends has undergone some chemical change or has disappeared. It is retained longest where the fibers are most numerous, as about the central canal.

2. Formaldehyde must be used as the fixative, either alone or combined with certain other reagents mentioned below. No other fixatives yet known penetrate so rapidly and preserve so well the chemical properties of the neuroglia-fibers. According to Weigert, who first fully recognized the value of formaldehyde in preserving neuroglia-fibers, the best strength to use is a 4 per cent. solution (*i. e.* a 10 per cent. solution of formaline, etc.). The pieces of tissue in which it is desired to stain the neuroglia-fibers must be cut very thin, never over $\frac{1}{2}$ cm. thick, and preferably thinner. With thicker pieces only the surface sections are of any value.

3. The tissues after fixation must be *mordanted,* so as to

render the staining more intense; without mordanting only a few of the fibers will stain.

4. Staining may be performed by modifications of Weigert's fibrin-stain or with phosphotungstic-acid hematoxylin.

The methods of staining neuroglia-fibers are given in the order of their publication. The second method (Weigert's) gives a more intense stain than the first, but has the drawback of staining degenerated nerve-fibers. The third method is particularly useful for class purposes, because a large number of sections can be stained all at one time. All of the methods are unsuccessful with the neuroglia-fibers of animals other than man.

A. **Differential Stain for Neuroglia-fibers** (*Mallory*).—1. Fix in a 4 per cent. aqueous solution of formaldehyde four days or more.

2. Place in a saturated aqueous solution of picric acid four to eight days.

Steps 1 and 2 may be combined by adding 10 parts of the 40 per cent. formaldehyde solution to 90 parts of the saturated aqueous solution of picric acid.

3. Transfer to a 5 per cent. aqueous solution of bichromate of ammonium for four to six days in the incubator at 37° C., or for three to four weeks at room-temperature. Change the solution on the second day.

4. Place directly in alcohol.

5. Imbed in celloidin.

6. Fasten sections to slide by means of ether-vapor.

7. Stain in aniline-gentian-violet fifteen to twenty minutes.

8. Wash off with normal salt solution.

9. Iodin solution, 1 : 2 : 100, for one minute, or a stronger solution for a few seconds.

10. Wash off with water.

11. Dry with filter-paper.

12. Decolorize in equal parts of aniline and xylol.

13. Wash off thoroughly with xylol.

14. Xylol balsam.

The neuroglia-fibers, fibrin, nuclei, and, to some extent, the red blood-globules, are stained blue. The other tissue-elements are colorless. By very faintly tinting the aniline-and-xylol mixture with fuchsin, which is readily soluble in aniline, the other tissue-elements are easily brought out, but the finer neuroglia-fibers are likely to lose their blue color.

Weigert's Method for Neuroglia-fibers.—*A.* Fix thin pieces of tissue, not over ½ cm. thick, in a 4 per cent. solution of formaldehyde for at least four days.

B. Mordant in the following solution for four to five days in the incubator or for eight days at room-temperature:

Acetate of copper,	5	gr.;
Acetic acid, 36 per cent. solution,	5	c.c.;
Chrome alum,	2.5	gr.;
Water,	ad 100	c.c.

Boil the chrome alum and water in a covered dish (the solution turns green in color), turn off the gas, add the acetic acid and then the acetate of copper; stir briskly until the latter is dissolved, then cool. The solution remains clear. If the directions and order of procedure are not followed exactly, a green precipitate will form.

(Steps 1 and 2 may be combined by adding 4 per cent. of formaldehyde to the above solution; change on the second day; harden eight days.)

C. Wash off in water; dehydrate in alcohol; imbed in celloidin.

D. Reduction of copper salt in sections:

1. Place the sections, which must not be over .02 mm. thick, in a ⅓ per cent. aqueous solution of permanganate of potassium for ten minutes.

2. Wash off with water.

3. Decolorize and reduce for two to four hours in the following solution, carefully filtrated:

Chromogen,	5	gr.;
Formic acid (sp. gr. 1.20),	5	c.c.;
Water,	ad 100	"

to 90 c.c. of which are added just before using 10 c.c. of a 10 per cent. solution of sulphite of sodium.

The sections lose their color in a few minutes, but are best kept in the solution as long as above directed.

The sections can now be stained in the manner to be described, but the color of the fibers will be more intense if the following steps are added, and a slight yellowish contrast-stain is obtained for the ganglion and ependymal cells and for the larger nerve-fibers. This step has one disadvantage, however: the connective-tissue fibers stain blue after it.

E. Further reduction of copper salt:

1. Wash twice in water.

2. Place sections in a carefully filtered saturated (5 per cent.) aqueous solution of chromogen over night.

3. Wash in water.

4. The sections are now ready for staining or may be preserved until wanted in—

80 per cent. alcohol,	90 c.c.
5 per cent. oxalic acid,	10 "

F. Staining of neuroglia-fibers:

1. Lift section from large dish of water on slide freshly cleaned with alcohol; blot with filter-paper (method recommended by Weigert for attaching sections to slide).

2. Stain in the following mixture:

Saturated solution of methyl-violet in 70–80 per cent. alcohol, (saturated with aid of heat; decanted when cold).	100 c.c.;
5 per cent. aqueous solution of oxalic acid,	5 "

The oxalic acid is added to render the preparations more lasting. The staining is practically instantaneous.

3. Wash off with normal salt solution.

4. Iodin solution: 5 per cent. iodid-of-potassium solution saturated with iodin. It is simply poured on and then off, as the reaction is instantaneous.

5. Wash off with water and blot with filter-paper.

6. Decolorize thoroughly in equal parts of xylol and aniline oil.

7. Wash repeatedly with xylol or the stain will not keep.

8. Canada balsam.

The sections keep better if exposed for from two to five days to diffuse light before being put away.

C. **Differential Stain of Neuroglia-fibers by means of Phosphotungstic-acid Hematoxylin** (*Mallory*).—1. Fix and mordant tissues in exactly the same way as in the first method given for neuroglia-fibers—viz.:

(*a*) Fix in 4 per cent. aqueous solution of formaldehyde four days.
(*b*) Saturated aqueous solution of picric acid four days.
(*c*) 5 per cent. aqueous solution of bichromate of ammonium four to six days in incubator or three to four weeks at room-temperature.
(*d*) Alcohol, celloidin, etc.

2. Place the sections in a $\frac{1}{2}$ per cent. aqueous solution of permanganate of potassium for fifteen to thirty minutes.

3. Wash in water.

4. One per cent. aqueous solution of oxalic acid fifteen to thirty minutes.

5. Wash in two or three changes of water.

6. Stain in phosphotungstic-acid hematoxylin for twenty-four hours to two or three days.

7. Wash quickly in water.

8. Dehydrate quickly in 95 per cent. alcohol.

9. Oleum origani cretici.

10. Xylol balsam.

Nuclei, neuroglia-fibers, and fibrin stain blue; axis-cylinders and ganglion-cells, pale pink; connective tissue, deep pink. The blue color is a little sensitive to strong light, and on prolonged exposure will fade to pink.

If a permanent isolated stain of the neuroglia-fibers is desired, place the sections (after staining as above directed in the phosphotungstic-acid hematoxylin and washing in water) in a 30 per cent. alcoholic solution of ferric chlorid for five to twenty minutes (rarely longer); then wash in water, and dehydrate as before. The nuclei, neuroglia-fibers, and fibrin stand out sharply of a clear blue color; everything else is decolorized or appears of a pale yellowish or grayish tint. The results obtained by this last step are practically identical with those obtained by either of the modified fibrin stains, and the method has the decided advantage of being applicable to any number of sections at once.

Degenerations of the Nervous System.—The same methods apply to the study of degenerations in nervous tissue that apply elsewhere, except in the demonstration of fat. Both myelin and fat reduce osmic acid, so that the ordinary test for fat in the hardened tissues fails. Marchi and Algeri, however, have shown that after myelin has been mordanted for eight days or over in Müller's fluid or other solution of the bichromates, it loses the property of reducing the osmic acid, while fat retains the property unimpaired. On this peculiarity is based their method for differentiating fat from myelin.

Marchi and Algeri's Method for Staining Fatty Degenerated Myelin-sheaths of Nerve-fibers.—1. Harden in Müller's fluid or in formaldehyde, followed by Müller's fluid, for eight days to three months.

2. Transfer tissue for five to eight days directly into the following solution:

Müller's fluid,	2 parts;
1 per cent. osmic-acid solution,	1 part.

3. Wash out thoroughly in water.
4. Dehydrate in alcohol.
5. Imbed in celloidin.
6. Clear in chloroform and mount in properly prepared chloroform balsam (see page 283).

EXAMINATION OF THE BLOOD.

The specific gravity of the blood varies but slightly and averages 1055. For clinical purposes the method of Hammerschlag is the best for estimating the specific gravity. The method depends upon the physical law that a body which remains suspended in a fluid must have the same specific gravity as that fluid. The fluid selected is a mixture of chloroform (specific gravity 1526) and benzol (specific gravity 0.889). A drop of blood does not mix with either fluid.

A small test-tube holding about 10 c.c. is half filled with a mixture of benzol and chloroform. This mixture should have the specific gravity of from 1050 to 1060. A drop of freshly-drawn blood is allowed to fall into this mixture, care being exercised that the drop falls directly into the fluid. Chloroform or benzol is added according as the drop of blood sinks to the bottom or floats on the surface of the fluid. It is necessary in adding either of the fluids to thoroughly mix them by gentle rotating movements without breaking the blood-drop. If the drop floats on the surface, it is better to add sufficient benzol to make it sink to the bottom, and then add chloroform until it becomes suspended in the fluid. Too large a drop of blood is liable to be broken up in mixing the fluids, and this must be avoided. When the drop remains suspended in the thoroughly mixed fluids the latter is filtered and the specific gravity tested.

Apparatus Used in the Examination of the Blood.—An accurate examination of the blood can be made only by the strictest observance of cleanliness and attention to details. It has been deemed wise, therefore, to devote the greater part of this article to a description of the various steps of the process for the benefit of those who are unfamiliar with the technique.

With this object in view it is not necessary to describe the numerous instruments which have been devised for examining blood or to refer to many of the staining fluids. The Thoma-Zeiss hemocytometer, or blood-counting instrument (Fig. 100), is the one which is generally employed to count the red and white blood-corpuscles, and consists of a glass slide on which the blood-corpuscles are counted and a pipette for mixing the blood and diluting fluid. The counting slide has a thin square plate of glass cemented on its surface: a circular opening in the center of this plate is nearly filled

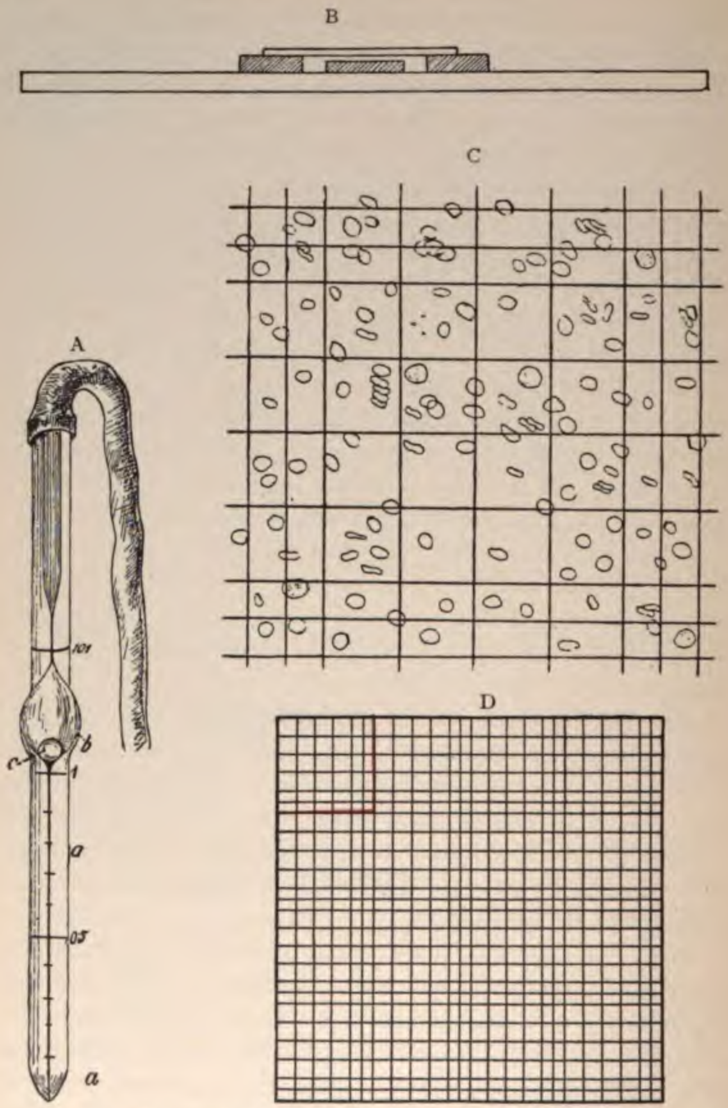

FIG. 109.—Thoma-Zeiss blood-counting apparatus (Limbeck): A, melangeur; *a*, capillary tube in which the blood is taken; *b*, chamber for mixing the blood with the diluting solution; *c*, glass ball to aid in mixing the blood with the diluting solution; B, cross-section of the chamber in which the blood is counted; C, section of the field on which the blood is counted, showing thirty-six squares; D, diagram of the whole field.

by a glass disc $\frac{1}{10}$ mm. thinner than the square plate which surrounds it. A series of horizontal and vertical lines on the surface of the disc divides it into squares, the sides of which are $\frac{1}{20}$ mm. long. Additional lines placed close together divide this surface into quadrants. Each quadrant contains sixteen of the small squares.

The pipette consists of a capillary tube which expands into an ovoid chamber above. The chamber contains a glass pearl, which assists in mixing the blood and diluting fluid. The capillary tube has a capacity of 1 mm. and is graduated to tenths. Above the ovoid chamber is a line marked 101 mm. A dilution of 1 to 100 or 1 to 200 is obtained by sucking the blood up to the mark 1. or 0.5, and the diluting fluid up to the mark 101.

A second pipette for counting the white corpuscles is graduated in such a way as to give a dilution of 1 to 10 or 1 to 20.

For clinical purposes the Von Fleischl hemometer (Fig. 110) is generally used to estimate the hemoglobin. This instrument is fairly accurate, but for percentages of hemoglobin below 50 per cent. it is safe to allow 5 per cent. for error in the apparatus. The instrument consists of a metal stand (*a*), a narrow wedge-shaped piece of colored glass (*b*), a hollow metal cylinder (*d*), and capillary tubes (*g*). The metal stand (*a*) is fitted with a flat stage above, in which is an aperture for holding the cylinder (*d*). On the under surface of this stage are two metal rims in which the glass wedge (*b*) fits. These rims are arranged so as to allow the glass wedge to move across the aperture by means of an adjustment screw. The glass wedge (*b*) is colored red, the degree of color becoming more intense toward the thick end. The glass is held in a metal frame, on one of whose sides parallel with the glass there is a scale of percentages from 0. to 120, corresponding to the intensity of the color in the glass. The metal cylinder (*d*) is divided into two equal compartments by a thin metal partition which extends from one end of the cylinder to the other. One end of the cylinder is closed by a piece of glass. The blood is collected in the capillary tube (*g*).

Preparation of Apparatus.—Three things are essential in order to make a satisfactory examination of the blood: the apparatus must be absolutely clean; the various stages in the process must be performed rapidly, because a very slight coagulation of the blood will interfere with any of the tests; and the work must be done accurately.

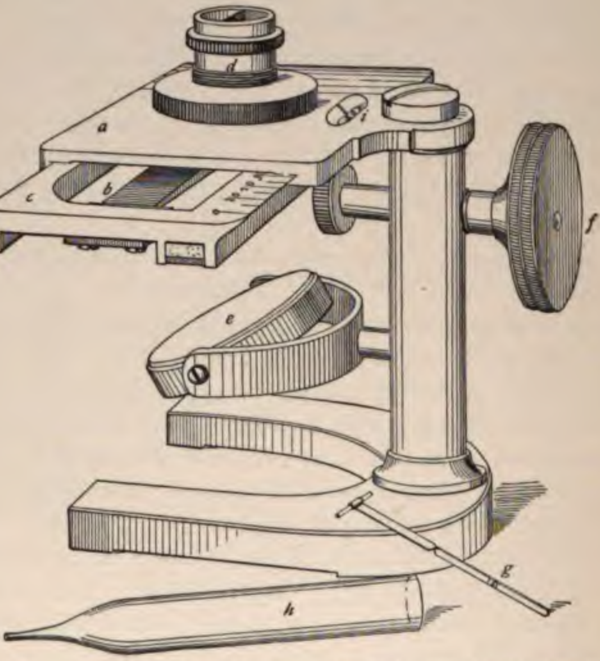

FIG. 110.—Von Fleischl's hemometer: *a*, stand; *b*, narrow wedge-shaped piece of colored glass fitted into a frame (*c*) which passes under the chamber; *d*, hollow metal cylinder, divided into two compartments, which holds the blood and water; *e*, white plate from which the light is reflected through the chamber; *f*, screw by which the frame containing the colored glass is moved; *g*, capillary tube to collect the blood; *h*, pipette for adding the water; *i*, opening through which may be seen the scale indicating percentage of hemoglobin.

Many of the details which follow may appear trivial to one who is unfamiliar with the methods employed in making blood-examinations. Experience will convince such an one to the contrary. All apparatus should be thoroughly cleaned and dried immediately after completing a blood-examination. The "mixing pipette" for estimating the blood-corpuscles can be readily cleaned by removing the rubber tubing and fixing it to the other end of the pipette, or a double atomizer bulb, such as is used on the Paquelin cautery, may be substituted. The pipette should be cleaned with water, alcohol, and finally with ether, until it is dry inside. If the alcohol fails to remove all of the staining fluid from the inside of the pipette and from the glass pearl, acid alcohol (hydrochloric acid 1 part, alcohol (70 per cent.) 100 parts) may be used. This should be followed by alcohol, and then by ether. The blood-counting slide may be cleaned with water. If any of the staining fluid adheres to the slide, it may be removed with alcohol. If alcohol is used to clean the counting slide, it must be used rapidly and the slide washed with water, because alcohol dissolves the cement by means of which the thin square of glass and the disc are fastened to the slide. The capillary tubes used for estimating the hemoglobin should be cleaned in the same way as the "mixing pipette." It has been found that the blood fills the capillary tube more readily if the latter is cleansed a second time with water directly before making the blood-examination. If this is done, it is necessary to thoroughly dry the tube afterward. This may be accomplished by removing most of the water and drying the remaining moisture by passing the tube rapidly through an alcohol flame. Care must be taken to avoid breaking it. The metal cylinder is cleaned by unscrewing the bottom and removing the glass. In this way the cylinder can be easily and thoroughly washed and dried. The cover-glasses should be kept in a solution of acid alcohol (see above). Before beginning a blood-examination a dozen or more of these cover-glasses should be cleaned with water, and then with alcohol, and dried; then they should be passed through an alcohol flame and spread on a piece of paper. The cover-glasses should not be touched with the fingers after cleaning them, but should be handled with forceps. New cover-glasses are covered with an oily glaze which prevents the blood from spreading. Moisture from the fingers may alter the shape of the red blood-corpuscles.

Never begin an examination of the blood until everything required for the entire examination is ready for use. A sufficient quantity of the diluting solutions must be poured into small dishes and placed, with the apparatus and cover-glasses, close at hand. If the preserving fluid contains a staining fluid, the solution must be filtered before using.

To **obtain the blood** the part from which it is taken should be cleaned with soap and water, alcohol, and finally with ether, and thoroughly dried. The blood is usually obtained from the lobe of the ear or from the end of the finger. The ear possesses distinct advantages over the end of the finger. It is not necessary to hold the ear to prevent its withdrawal; the operation is less painful, and the blood is obtained more easily because the epidermis is thinner. These advantages are especially evident in infants and young children. In children it is almost impossible to keep the fingers extended without exerting pressure. Pressure on the part from which the blood is withdrawn must be avoided. It has been ascertained that moderately firm pressure on the finger is capable of diminishing the number of red corpuscles in a cubic millimeter by increasing the quantity of serum. The free border of the lobe of the ear is preferable to the anterior surface, because the border is convex. This is of advantage in making cover-glass preparations from small drops of blood. A narrow-bladed knife, similar to a tenotomy-knife, makes a relatively wide and shallow opening, and is preferable to a needle.

The hemoglobin test must be performed in a dark room by means of a yellow light—lamp, gas, or candle light. Before obtaining the blood fill one of the divisions of the cylinder one-fourth full with distilled water; then partly fill the pipette provided for this purpose, and place it close at hand ready to wash out any blood which may remain in the capillary tube, and thus prevent coagulation. The capillary tube is held horizontally, and touched slightly on the side of a *freshly* drawn drop of blood. The end of the tube should never be dipped deeply beneath the surface of a blood-drop. If the tube is perfectly clean and no coagulation has occurred in the drop of blood, the latter will instantly fill the tube. Sometimes it is necessary to slightly withdraw the end of the tube, without, however, permitting it to entirely leave the surface of the blood. Observe that the tube is exactly filled with blood and that the ends show neither convexity nor concavity. Any blood adhering to the end of the tube or to its outer surface must be quickly wiped off. Rapidly submerge the tube in the water and move it quickly to and fro several times, and with as little delay as possible wash out the remaining blood by means of the pipette. Fill the chamber three-fourths full with water and stir briskly with the metal handle of the capillary tube. Be careful to stir in the corners where the partition joins the sides. The blood should be equally diffused throughout the fluid when the mixing has been well done, and there should be no coagula in it. When this has been done fill the cylinder to the level of the diaphragm; the other division of the cylinder is then filled to the level of the diaphragm with distilled water. The surface of the fluid in each side must be perfectly flat. This can be readily ascertained by looking across the surface of the fluid with the eyes on a level with the top of the cylinder. A convexity or concavity of the water side can be easily obviated. A convexity on the side which contains the blood should be avoided. When the cylinder is in position, the division containing the distilled water is over the colored glass. The reflector should be arranged to throw as much light as possible when the percentage of hemoglobin is high; but when the hemoglobin is much diminished, less light will give more exact results. To perform the test one eye should be closed and the face shielded from direct light by holding a piece of paper between the face and the lamp. The instrument should be arranged so that the observer stands at the side of it and looks directly down into the fluid. This is done so that the images of both divisions of the cylinder are thrown upon the retina side by side, and not one above the other. This permits a more accurate comparison of colors to be made. By means of the adjustment screw move the colored glass from a point where the glass is paler than the diluted blood to a point where the color of the glass and of the blood match. Then read the percentage on the scale, and control the result by moving the colored glass from above the point where the colors match downward, to see if the same result is obtained; pause a moment and repeat the test. More accurate results are obtained by resting the eye several times than by looking for a long time.

In order to **estimate the number of red corpuscles** in a cubic millimeter of blood it is necessary to use a diluting fluid. It is desirable that this fluid should be of such a character that the corpuscles may not change their form or lose their color. Various fluids are used. Toison's fluid possesses the advantage of staining the white corpuscles. The formula of this fluid is as follows:

Distilled water,	160	c.c.;
Neutral glycerin,	30	"
Sodium sulphate, pur.,	8	grams;
Sodium chlorid,	1	gram;
Methyl-violet, 5 B,	0.025	"

This fluid must be filtered each time before using. A drop of blood is required of sufficient size to more than fill the capillary tube of the "mixing pipette." If the drop is too small, it is difficult to prevent the entrance of air and coagulation of the blood in the tube in the interval of obtaining a second drop of blood. It is advisable, therefore, to perform this test before estimating the hemoglobin or making the cover-glass preparations. While filling the capillary tube of the pipette care must be exercised to keep the point of the pipette beneath the surface of the blood-drop to prevent the entrance of air. The blood is sucked up to the mark .5 or 1., and then the tip of the tongue is pressed firmly over the hole in the mouth-piece. This prevents the blood-column from sinking or air from entering below while the tip of the pipette is being wiped and immersed in the diluting fluid.

This part of the test requires the utmost precision and avoidance of delay. It is necessary, therefore, to keep the eyes constantly fixed on the capillary tube in order to note any variation in the blood-column. Rapidly wipe the tip of the pipette to remove the blood from the outside, and then immerse the tip in the diluting fluid. Suck the fluid up to the mark 101, close the ends of the pipette with the thumb and middle finger, and shake the pipette for two minutes. If the ends of the pipette are not completely closed during this process, some of the fluid will escape. At the end of two minutes allow two drops to escape from the pipette before examination, because the fluid in the capillary tube is unmixed with blood. Then allow a drop to escape upon the central part of the counting slide. This drop should completely fill the depression after the cover-glass has been applied. A little practice is necessary in order to estimate the size of the drop required. A moderately thick cover-glass should be slid over or carefully laid upon the square raised surface, and pressure applied to the edges until the Newton color-zone can be seen between the cover-glass and the square raised surface beneath. Never press on the center of the cover-glass. Allow the blood-corpuscles to settle a minute or two before counting.

The corpuscles are estimated as follows: One side of a small square is $\frac{1}{20}$ mm. long; the enclosed square surface is $\frac{1}{400}$ mm. The distance between the cover-glass and the disc is .1 mm. which gives a cubic capacity of $\frac{1}{4000}$ c.mm. for each square. To estimate the number of corpuscles in 1 c.mm. of blood, multiply the number of corpuscles counted by 4000, and then by the number representing the amount of dilution, 100 or 200 as the case may be, and divide the result by the number of squares counted.

$$\frac{\text{Corp.} \times \text{dilution} \times 4000}{\text{Squares counted}} = \text{corpuscles in 1 c.mm.}$$

To avoid counting any of the corpuscles twice, always begin at the upper left-hand square of a quadrant and count four squares downward. Count all the corpuscles which touch the upper and left-hand lines of a square, together with the corpuscles in the square. Never count the corpuscles touching the right-hand or lower double lines of a quadrant. In order to make an accurate count it is necessary to count at least 1200 red corpuscles.

If air-bubbles are present when the cover-glass is applied, it is necessary to clean the slide and use a fresh drop of the diluted blood. Before beginning the count examine the various quadrants with a low-power objective, to see if the corpuscles are evenly distributed. If they are not, it will be because the blood is not thoroughly mixed, and the slide should be washed and the pipette well shaken. Before examining a second drop of the diluted blood shake the pipette for two minutes as before. The results of three drops should be averaged.

There is a special pipette for counting the white corpuscles, graduated so as to give a dilution of 1 to 10 or 1 to 20. The white corpuscles are estimated in the same way as the red corpuscles, except that the dilution 10 or 20 is substituted for 100 or 200. This necessitates a fresh drop of blood. For a diluting fluid for counting the white corpuscles a $\frac{1}{3}$ or $\frac{1}{2}$ per cent. solution of acetic acid may be used. This solution destroys the red corpuscles. A little gentian-violet solution may be added to the acetic acid if it is desired to stain the white corpuscles.

It is possible to estimate both the white and red corpuscles in the same drop of blood quite accurately. In order to do this it is necessary to calculate the number of squares which would be contained in the entire ruled surface outside of the portion which is divided into quadrants. This may be done by means of a micrometer eye-piece and a movable stage. The number has been found to be about 2000, although there may be some slight variation in each slide. This enables one to count 50 white corpuscles or more in each drop by counting all the corpuscles within the lines. For calculating the number of corpuscles one would divide by the estimated number of squares counted, just as in determining the red corpuscles. The average of three drops gives quite accurate results and saves much time and trouble.

Cover-glass Preparations.—The blood must be spread extremely and uniformly thin. If this is done, the blood dries very quickly, and the red blood-corpuscles retain their shape and are not crowded together and lying over one another. To obtain such a result it is essential that the cover-glasses should be absolutely clean; that there should be no delay in bringing the cover-glass which has the drop of blood on its surface in contact with a second cover-glass; and that the drop of blood should be quite small. The following method gives the best results: The procedure is rendered much easier if some one is present to assist. This person places a finger beneath the lobe of the ear in order to raise it slightly without pressing upon it, and with a clean compress wipes away the blood as fast as it flows with a quick motion of the hand. This is done to prevent coagulation, which occurs very quickly, and prevents the drop of blood from spreading between the cover-glasses. It takes a little time for the blood to spread, the cover-glasses to be separated and laid down, and fresh ones picked up; and if, during this time, some one wipes away the blood as fast as it flows, much better preparations are obtained. If, in spite of this,

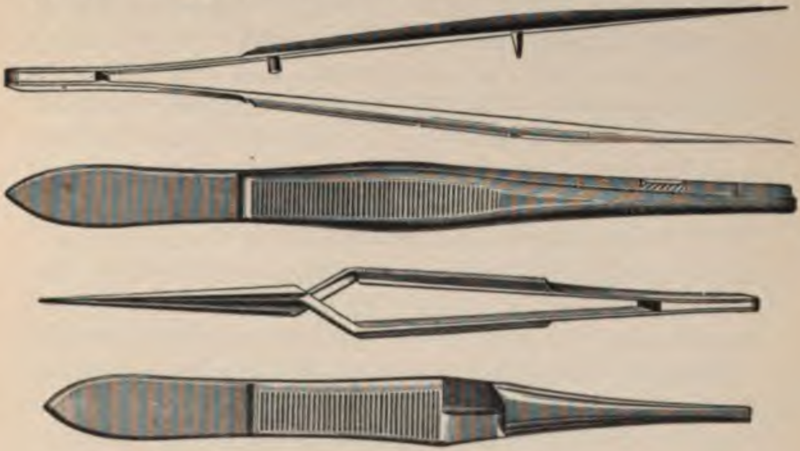

FIG. 111.—Forms of forceps for holding cover-glasses.

as often happens, the blood coagulates about the opening, one end of the compress can be slightly moistened with water and passed over the opening and the surface dried quickly. The blood then flows freely again. A drop of blood a little larger than a pin-head is sufficient. Grasp the edge of the cover-glass with a pair of spring forceps, pick up a second cover-glass with a pair of plain forceps. Both pairs of forceps must be especially prepared by having the inner surfaces of the points ground smooth. The cover-glass in the spring forceps is held horizontally just below the ear, and the other cover-glass, held with the other forceps, is touched lightly on the blood and immediately dropped on the first one. If the cover-glasses are dry and clean and the blood has not begun to coagulate, it spreads at once in a thin film between the glasses. The glasses are then drawn apart with a rapid sliding motion by means of the forceps, waved in the air a few seconds, and laid down with the blood-surface uppermost. The layer of blood cannot be too thin, but it can easily be too thick. The cover-glasses should never be pressed together to make the blood spread. Considerable practice is required before one becomes proficient. The specimens may be fixed by heat or by a mixture of alcohol and ether, equal parts. The best method is to put the cover-glasses for twelve hours in a thermostat at a temperature between 110° and 120° C. (Ehrlich's method). This is objectionable on account of the time and apparatus required. A practical modification of this method is to heat the cover-glasses on a brass plate for an hour at a point on the plate where water boils. The plate should be about $\frac{1}{8}$ of an inch thick and from 15 to 18 inches long. It should be heated from one end to a constant temperature. Test the degree of heat with drops of water and select a part where the water boils. At a point nearer the flame it will be found that the water sputters and rolls about, indicating too high a temperature. After putting the cover-glasses, with the blood-side uppermost, upon the selected point, it is necessary to test the degree of heat from time to time, and perhaps to shift the cover-glasses.

A shorter and easier method, not so satisfactory, is to fix the specimens by immersing them for from thirty minutes to an hour in a mixture of absolute alcohol and ether, equal parts. This gives good results, as a rule.

A very simple and quick method of fixation has recently been devised by Dr. W. F. Whitney. Fix the preparations with Zenker's fluid, in which the acetic acid is replaced by nitric acid, for ten to fifteen seconds.

Bichromate of potassium,	2.5 g.;
Corrosive sublimate,	5.0 g.;
Sulphate of sodium,	1.0 g.;
Nitric acid,	5.0 c.c.;
Water,	ad 100.0 c.c.

Wash off with water; drain or blot off excess of water, and stain in the usual way. Ehrlich's triple stain is particularly recommended.

The Elements of the Blood.—Red corpuscles, white corpuscles, and hematoblasts. The red blood-corpuscles (erythrocytes) are biconcave discs. The diameter of a red blood-corpuscle in human blood is the same for male and female, and averages 7.8μ. The red corpuscles have a pale-yellow color with a faint tinge of green; they are homogeneous, highly refractive, and have no nuclei (normally). Outside the circulating blood the corpuscles rapidly undergo alterations in their shape and size. These changes may be prevented or hindered, for purposes of study, by appropriate methods of fixation and preservation. The average number of red corpuscles in 1 cubic millimeter of human adult blood is from four million to five million. Under abnormal conditions the red corpuscles vary in size, shape, and number, and a small proportion of them may have nuclei.

1. Variations in the shape (*poikilocytosis*) are of frequent occurrence. Some of the corpuscles may be pear-shaped or bottle-shaped; others may be shaped like saddle-bags or have projections. Variations in the size of the erythrocytes are also common. They all retain the central depression, notwithstanding the variations in shape and size.

2. Abnormally large erythrocytes, varying from 10 to 14μ in diameter and without nuclei, are called megalocytes. Very small erythrocytes, varying from 2 to 5μ in diameter, are called microcytes. The microcytes contain hemoglobin, but do not have the central depression.

3. Nucleated red corpuscles (erythroblasts) occur in two forms, the so-called normoblasts and the megaloblasts or gigantoblasts. A normoblast is the same size as a normal corpuscle, but contains a single relatively large nucleus. The nucleus is commonly situated in the centre of the corpuscle, but it may lie in the periphery, and takes a more intense stain than the nucleus of any other corpuscle. Mitosis is observed frequently. Free nuclei are found often. A megalo- or gigantoblast is from three to five times as large as a normal red corpuscle, and contains a relatively large round nucleus in the center. The intensity with which this nucleus stains is midway between that of the normoblast nucleus and the nuclei of the white corpuscles.

4. The so-called *anemic degeneration* of the red corpuscles is less commonly met with than the preceding changes. When cover-glass-preparations are stained with eosin-hematoxylin or eosin-methylene-blue, the red corpuscles, instead of staining a bright eosin-red, take a muddy-looking, darker stain. This appearance is assumed to be due to degenerative changes in the stroma of the corpuscles, which cause the hemoglobin to become stained to some extent by the nuclear stain in addition to the eosin.

The *white corpuscles* (leucocytes) differ in size, in the size and shape of their nuclei, and in the granules which are contained in the protoplasm. They have been classified in various ways, depending upon their supposed origin and upon the granules which they contain.

Ehrlich differentiates five different kinds of granules found in the human blood: the α or eosinophilic granules; the β or amphophilic; the δ and γ or basophilic granules; and the ε or neutrophilic granules.

The α or eosinophilic granules are coarse, round or oval, highly refractive granules which have an affinity for the acid aniline stains, especially eosin.

The β or amphophilic granules have an affinity for both acid and basic stains. They occur very rarely in human blood, but are common in the blood of certain animals.

The δ and γ granules are finer and less refractive granules which have an affinity for basic stains. The γ granules are nearly as large as the eosinophilic granules, but are not so refractive. The δ granules are much finer and not very refractive, and are very numerous in the cells in which they occur.

The ε or neutrophilic granules are much smaller, more numerous, and less refractive than the eosinophilic granules, and have an affinity for neutral stains. They occur only in human blood.

Morphologically, there are five varieties of white corpuscles. They are—the small mononuclear cell or lymphocyte; the large mononuclear cell; the transitional large mononuclear cell; the neutrophile; and the eosinophile. The small mononuclear cell or lymphocyte is slightly larger than a red corpuscle, and has a single round nucleus surrounded by a narrow strip of protoplasm which contains no granules. The nucleus stains more intensely than the nuclei of the other white corpuscles.

The large mononuclear cell is much larger than the lymphocyte, and contains a large, oval or round nucleus surrounded by a wide zone of protoplasm. The nucleus takes a fainter stain than that of the lymphocyte.

The transitional large mononuclear cell differs from the large mononuclear only in having a saddle-bag-shaped nucleus. Variations in size are frequently observed in the small mononuclear as well as in the large mononuclear cells, so that under some circumstances it is difficult to distinguish to which type of cell a given cell belongs.

The neutrophile or polymorpho-nuclear leucocyte is about three times as large as a red corpuscle. This cell is the so-called polynuclear leucocyte found in pus. The nucleus, as the name implies, is more or less subdivided, and often resembles various letters, as S, Z, V, M, etc. The nucleus is surrounded by a relatively large amount of protoplasm which is more or less completely filled with fine neutrophilic granules.

The normal eosinophile resembles the neutrophile in size and in the character of its nucleus. It differs from the neutrophile, at least chemically, by containing the eosinophilic α granules.

The large mononuclear, transitional, and neutrophilic cells are regarded as the same cell in different stages of development. The younger form is the large mononuclear, and the oldest is the neutrophile. During the process of "ripening," as it is called, the fine ε granules are formed in the protoplasm, and the nucleus becomes more or less subdivided.

The so-called *mastzellen*—that is, cells which contain basophilic granules—were supposed by Ehrlich to originate from fixed connective-tissue corpuscles and from the spleen, and not to be present normally in the blood. Later investigations show that an occasional *mastzelle* may be found in normal blood.

Markzellen, or myelocytes, is the name applied to large mononuclear cells containing neutrophilic, and sometimes eosinophilic, granules. These cells are present in the medullary cavity of the long bones, but are never found in the blood normally.

Hematoblasts (*blut-plättchen*) are small round or oval discs from 1.5 to 3.5μ in diameter. They do not contain hemoglobin and have no central depression. They are so rapidly disintegrated on exposure to the air that it is necessary to use special precautions in order to preserve them. The best way to study them is to put a drop of some preserving fluid on the skin from which the blood is to be obtained, and then puncture the skin through the fluid. A 1 per cent. aqueous solution of osmic acid can be used, or, if it is desired to stain them, 1 part of methyl-violet in 5000 parts salt solution may be used.

Methods of Staining.—Of the many staining fluids which have been employed to differentiate the white corpuscles, it is necessary to mention only those which are commonly used and which have been found to answer all purposes.

Ehrlich's triple stain possesses the advantage of staining both the eosinophilic and neutrophilic granules in addition to the nuclei. The formula is as follows:

Orange G, saturated aqueous solution,	120 to 135 c.c.;
Distilled water,	100 "
. . . .	
Acid fuchsin, saturated aqueous solution,	65 "
Distilled water,	100 "
Absolute alcohol,	100 "
. . . .	
Methyl green, saturated aqueous solution,	125 "
Distilled water,	100 "
. . . .	
Absolute alcohol,	100 "
Glycerin,	100 "

The various ingredients are prepared separately as indicated by the dotted lines, and are afterward mixed gradually. The mixture must stand for several weeks before using. It is advisable to withdraw by means of a pipette some of the staining fluid from the middle portion without disturbing the bottom.

The cover-glass preparations should be stained from six to eight minutes, washed thoroughly with water, dried, and mounted in Canada balsam. The neutrophilic granules are stained violet; the eosinophilic, a bright red; the nuclei of the neutrophilic and the eosinophilic cells are a greenish-blue; the nuclei of the lymphocytes, a deep blue; the nuclei of the large mononuclear cells, a pale blue; the red corpuscles, copper color: and the nuclei of the red corpuscles, if any be present, a more intense blue than the nuclei of the lymphocytes. For some unexplained reason this stain is not always uniform in its action.

It is sometimes difficult to distinguish a nucleated red corpuscle from a lymphocyte. It is well to remember, therefore, that the nuclei of red corpuscles stain more intensely than other nuclei, and have very sharply defined outlines, and by careful focussing it is seen that the surrounding stroma is stained the same color as the other red corpuscles.

Ehrlich's Hematoxylin-eosin Stain.—

Distilled water,		
Alcohol,		
Glycerin,	*āā.*	100 grams;
Hematoxylin,		4–5 "
Acetic acid,		20 "
Alum in excess.		

The mixture remains from four to six weeks in the sun, and then about 1 per cent. of eosin is added. Stain for from twelve to twenty-four hours in a covered glass dish in the sun. Wash thoroughly in water, dry, and mount in Canada balsam.

The red corpuscles are stained red, with at times a tinge of orange; the nuclei of the red corpuscles, a deep black; the protoplasm of the leucocytes, a light lilac; the nuclei, a dark lilac; the eosinophilic granules, a bright red; the nuclei of the lymphocytes, black, with not quite so deep a tinge as the nuclei of the red corpuscles. The protoplasm scarcely stains.

Eosin and Methylene-blue Stains.—Separate stain. The eosin solution, a $\frac{1}{2}$ per cent. alcoholic solution, is heated and the cover-glasses are stained in it from two to three minutes, and are then stained in a saturated aqueous solution of methylene-blue for from two to three minutes; thoroughly washed, dried, and mounted in Canada balsam.

The red corpuscles are stained eosin red; the nuclei of the red corpuscles, a deep-blue; the nuclei of the leucocytes are stained a light blue; the eosinophilic granules, a bright red.

Chenzinsky-Plein Solution.—Saturated aqueous solution of methylene-blue, 40 c.c.; ½ per cent. (in 70 per cent. alcohol) eosin solution, 20 c.c.; distilled water, 40 c.c.

The best results are obtained by staining the specimens for twenty-four hours. A fairly good stain may be obtained by heating the solution fifteen minutes. The red corpuscles are stained eosin red; the eosinophilic granules, a bright red; and the nuclei, blue.

The basic granules may be stained by a concentrated solution of methylene-blue. The specimens should remain in the solution from five to ten minutes.

METHODS OF FIXING AND EXAMINING SPECIAL ORGANS AND TISSUES.

TISSUES which are to be hardened should be obtained as fresh as possible. For this reason autopsies rarely furnish such perfect material as is obtainable from experimental lesions in animals or from surgical operations. Still, most of the pathological material comes from autopsies, and it is encouraging to know that very good work can often be done with tissues not fixed until twenty-four hours or even more after death. The most valuable autopsies are those which are freshest, and in which but one etiological factor has been concerned, so that the relation between the cause and the lesion produced is uncomplicated and can be readily grasped and understood.

The choice of the proper fixing reagent varies with the tissue, the lesion, and the use to which the material is to be put. For simple diagnosis alcohol is usually sufficient. For special investigations other fixatives may be required. In general it may be said that alcohol is best suited for bacteria and for many micro-chemical color reactions; Zenker's or Orth's fluids for red blood-globules, nuclear figures, and protoplasm; formaldehyde for the central nervous system. More attention will be paid to this point in the consideration of the separate organs and tissues.

It is important that pieces of tissue for histological study should be placed in the proper fixative as soon after the removal of the organs from the body as possible, so that the surface will not dry or the blood and other fluids escape from the vessels. Do not wash off the surface with water. The tissues should almost invariably be cut into thin slices, not over 4 to 8 mm. thick. For the finer fixatives 2 mm. should not be exceeded.

Frozen sections of fresh tissues will often show whether the material is worth saving, and suggest what fixatives had best be used—such as Flemming's solution, for instance, if fat is present.

Acute Inflammatory Exudations; Granulation-tissue.—The elements in acute inflammatory exudations which require preservation are chiefly polynuclear leucocytes, serum, fibrin, and red blood-globules. The best general fixative for them all is Zenker's fluid. It not only preserves perfectly the characteristic nuclei of the leucocytes, but also the protoplasm which stands out sharply in contrast-staining with eosin. The albumin of the serum is coagulated into a finely granular material. The fibrin and red blood-globules stain brilliantly with eosin. Alcohol is generally preferred for the fixation of any organisms associated with the exudation, but after fixation in Zenker's fluid they can be perfectly stained with Unna's alkaline methylene-blue solution. Occasionally other fixatives, such as Flemming's or Pianese's, will be found useful, especially for the study of any attendant degenerations.

Zenker's fluid will also be found invaluable for fixing granulation-tissue where a similar exudation is generally combined with new-formed blood-vessels and connective tissue.

For the study of the plasma-cells which early make their appearance in granulation-tissue hardening in alcohol is generally advisable, although Zenker's fluid is often to be preferred, and for eosinophiles is indispensable.

Lung.—In the preservation of the lungs it is important to save portions that have not been squeezed, so that the relations of the exudations may not have been changed or the alveoli compressed. Thin slices are usually preferable to cubical pieces, and should be cut with a very sharp knife, so as not to compress the tissue, and dropped immediately into the fixing fluid, before the contents of the bronchi and of small cavities have had time to run out. An emphysematous lung is so delicate that it is usually better to inject a whole lung through the bronchi with the fixing fluid or to snip out small pieces with scissors. Zenker's fluid and alcohol are the most useful fixatives.

Spleen.—The spleen-pulp may be examined by means of dried cover-slip preparations in the same way as described for the blood. Material can be obtained immediately after death by means of a long trocar thrust into the spleen. Teased preparations can be examined in salt solution. The spleen-tissue is well preserved in Zenker's fluid if cut into thin pieces not over 2 to 4 mm. thick. Paraffin sections are to be preferred when convenient, so that the thinnest possible sections may be obtained.

Bone-marrow may be examined in the same way as spleen-pulp in cover-slip preparations, or thin slices may be hardened in Zenker's fluid or some other fixative such as formaldehyde for study by means of sections. Eosin followed by alkaline methylene-blue solution is strongly advised.

Kidney.—The choice of fixing reagents varies largely with the lesions present. Zenker's fluid is advisable for general histological purposes, for blood, and for hemoglobin; alcohol for bacteria, amyloid, hemosiderin; Flemming's for fat; boiling water for albuminous exudations. The pieces of tissue preserved should include both cortex and pyramids.

In cases of chronic nephritis the capsule should not be peeled from those parts kept for microscopical purposes.

Paraffin imbedding is generally to be preferred for the kidney, especially when lesions of the glomeruli are present.

For the simple demonstration of fat, teased preparations or frozen sections of fresh material can be mounted in water and treated with acetic acid or stained in sudan III.

Gastro-intestinal Tract.—Portions of the stomach or intestine should be hardened as soon after death as possible for satisfactory study, because the gastro-intestinal tract so rapidly undergoes post-mortem changes. It has been recommended in appropriate cases, where an autopsy is allowable, to inject the stomach with the desired fixing solution by means of a rubber tube as soon after death as is permissible. Under no circumstances should the surface of the intestine or stomach be washed with water. Use either normal salt solution or some of the fixing solution. It is important to keep the tissue flat while hardening. This can usually be done by laying it with the peritoneal surface down on thick filter-paper, to which it readily sticks. Sometimes it is necessary to pin the specimens down at the edges on flat pieces of cork. Do not let the surface dry before the specimen is placed in the fixing solution. Zenker's fluid can be highly recommended as a fixative, but alcohol is sometimes to be preferred.

Liver.—For fat the liver is examined fresh in teased preparations or frozen sections, or after hardening in Flemming's or Marchi's solution, by means of celloidin or paraffin sections.

For obtaining the iron reaction with hemosiderin in cases of pernicious anemia, and for the reactions of amyloid, harden in alcohol.

For general histological study Zenker's fluid will be found exceedingly useful.

The bile-capillaries may be demonstrated by means of Golgi's silver method.

Boehm's directions for it are as follows:

1. Harden pieces of liver 1 cm. in diameter for seventy hours or more in

3 per cent. solution of bichromate of potassium,	4 parts;
1 per cent. solution of osmic acid,	1 part.

2. Transfer for twenty-four to forty-eight hours to a $\frac{3}{4}$ per cent. solution of nitrate of silver.
3. Wash in water.
4. Harden and cut.

The bile-capillaries appear dark brown on a yellow ground.

Bone and Cartilage.—Excellent work can be done after hardening in alcohol, and fixation in it is generally recommended for all infectious processes in bone. The histological structure is, however, better preserved in Zenker's or Orth's fluid. In decalcifying bone, after proper fixation, thin pieces should be taken, not more than 2 to 4 mm. thick, so that the process may be finished as quickly as possible. While tubercle bacilli will stain readily after being twenty-four or even forty-eight hours in 5 per cent. nitric acid, it is impossible to stain them after they have been subjected to the same strength of nitric acid for four days. (For details in regard to decalcification see page 252.)

Celloidin is preferable to paraffin for imbedding. Besides a simple stain with alum-hematoxylin, double stains of the latter with neutral carmine or eosin are sometimes advantageous. The best pictures with carmine as the contrast-stain are obtained by staining first in alum-hematoxylin, washing twelve to twenty-four hours, and then staining in the neutral carmine. The carmine stains decalcified bone and osteoid tissue red. Phosphotungstic-acid hematoxylin will sometimes be found useful, especially when cartilage is present, because it stains the intercellular substance both of bone and of cartilage pink, while the nuclei are stained blue. The ground substance of cartilage, especially in new-growths, often stains so intensely with alum-hematoxylin that the nuclei are quite obscured. For the same reason chlorid of iron hematoxylin is often useful because it does not stain the ground substance.

The following method is recommended for differentiating cartilage from bone:

Schaffer's Safranin Method.—Decalcify with nitric acid.

1. Stain sections a half to one hour in an aqueous solution of safranin, 1 : 2000.

2. Wash in water.

3. Place for two to three hours in a $\frac{1}{10}$ per cent. solution of corrosive sublimate.

4. Examine in glycerin, or, if permanent specimens are desired, pass very quickly through alcohol, blot with filter-paper, further dehydrate, and clear for a long time in bergamot or clove oil, and mount in xylol balsam. This is a double stain: cartilage, orange; bone, uncolored; connective tissue and marrow, red.

None of the methods above given have proved reliable in the study of rickets and of osteomalacia for differentiating osteoid from true bone-tissue. In important cases, therefore, it is advisable to use an old knife, and to cut sections of the undecalcified tissue after imbedding thoroughly in celloidin.

Schmorl's methods[1] of demonstrating the lacunæ and canaliculæ of bone in sections can be highly recommended.

Method A.—1. Fix preferably in Müller's fluid, formaldehyde, or Orth's fluid; do not use corrosive sublimate solution.

2. Decalcify by the slower methods—namely, Ebner's or Thoma's, or in Müller's fluid 100 c.c. plus nitric acid 3 c.c.

3. Imbed in celloidin; paraffin is objectionable.

4. Place the sections for at least ten minutes in water to get rid of the alcohol.

5. Stain for five to ten minutes or longer in saturated solution of thionin in 50 per cent. alcohol, 2 c.c., water, 10 c.c., or in Nicolle's carbolthionin solution.

6. Wash in water.

7. Place in a saturated aqueous solution of picric acid for one-half to one minute.

8. Wash in water.

9. Place in 70 per cent. alcohol for about five to ten minutes until no more dense clouds of color are given off.

10. Dehydrate in 95 per cent. alcohol.

11. Clear in oleum origani cretici.

12. Xylol balsam.

Bone substance yellow to yellowish-brown; bone lacunæ and canaliculæ dark brown to black; cells red. Fat-cells after fixation in Müller's fluid reddish violet. Osseous tissue stains a deeper yellow than osteoid tissue. Canaliculæ stain in osseous tissue, but not in osteoid tissue unless the thionin solution is made alkaline by the addition of 1 or 2 drops of ammonia. (This solution cannot be recommended for general use.)

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[1] Schmorl: *Centralblatt für allg. Pathologie*, 1899, x., 745.

This method is not a true stain, but resembles Golgi's method; a precipitation of coloring-matter takes place in the lacunæ and canaliculæ; it also takes place to a considerable extent in other narrow spaces in the tissues, and often is very disturbing. It can be gotten rid of to some extent without injury to the stain by leaving the sections in step 8 in the water for half an hour. The canaliculæ are now usually brownish red to red, and the bone substance blue to colorless. In this case it is often best to stain the sections first in alum hematoxylin to bring out the nuclei.

Method B gives good results with the bones of children only. 1. Harden in Müller's fluid or in Orth's fluid, followed by Müller's for six to eight weeks, or for three to four weeks in the thermostat; take very thin pieces of tissue.

2. Wash off in water, and decalcify in Ebner's solution.

3. Wash thoroughly in running water.

4. Harden in alcohol; imbed in celloidin; cut sections very thin.

5. Stain in Nicolle's carbolthionin, or better in the alkaline (NH_4OH) thionin solution given above, for three minutes.

6. Transfer to a saturated aqueous solution of phosphotungstic or phosphomolybdic acid (use glass or platinum needle) for a few seconds or longer. The sections become blue, green, or gray in color.

7. Water five to ten minutes until they acquire a sky-blue color.

8. Place in dilute ammonia (1–10) for three to five minutes to fix the color.

9. Transfer directly to 90 per cent. alcohol; change several times to get rid of the ammonia.

10. 96 per cent. alcohol.
11. Clear in carbol xylol.
12. Xylol balsam.

If the ground-substance is stained too deeply by the alkaline thionin solution, treat the sections with acid alcohol for five minutes, followed by water before dehydrating. The borders of the lacunæ and canaliculæ stain bluish black; the ground-substance of bone clear to greenish blue; cellular elements a diffuse blue color. In rachitic bones the canaliculæ are brought out only in osseous tissue.

Skin.—Much of the material for the study of lesions of the skin is obtained during life by means of a Mixter punch or with the knife or scissors. Fixation in absolute alcohol is often advisable, especially when it is desired to stain bacteria, mastzellen, plasma-cells, and elastic fibers. The staining methods for these tissue-elements will be found on pages 301–308. For Unna's innumerable stains for degenerated connective-tissue fibers, elastic fibers, etc. the reader is referred to his numerous articles on technique in the *Monatsheft. f. prakt. Dermatologie* during the last half-dozen years.

For many skin-lesions, especially those in which blood-vessels play a more or less prominent part, Zenker's fluid is advisable. For finer histological work Pianese's fixatives and special stains are recommended.

In the examination of hairs or scales of epidermis for bacteria and fungi it is important first to remove the fat from them by means of equal parts of alcohol and ether. They are then examined in 40 per cent. caustic potash, which, by clearing up the cells, brings out the organisms and spores quite distinctly. Heating the potash over a small flame hastens the process, but is a somewhat risky proceeding; soaking in the solution over night is better. Examine the preparation with most of the light excluded.

Preparations may be made in certain cases by touching the cover-slip to the surface of the lesion, drying, and passing through the flame. After removing the fat by means of alcohol and ether, stain as with ordinary cover-slip preparations.

Unna's method is to rub up the scales of epidermis in a little glacial acetic acid between two slides, which are then drawn apart and quickly dried over the flame. After removing the fat by means of alcohol and ether the slide preparations are stained in borax-methylene-blue.

For staining the various vegetable parasites of the skin Malcolm Morris recommends the following method, which he claims is the best one yet devised, as it avoids the use of the hydrate of potash:

1. Ether, or alcohol and ether equal parts.
2. Stain in a solution of 5 per cent. gentian-violet in 70 per cent. alcohol, five to thirty minutes.
3. Iodin solution, one minute.
4. Aniline, or aniline plus 2 to 4 drops of nitric acid.
5. Aniline.
6. Xylol.
7. Xylol balsam.

The most suitable medium for the growth of the various ringworms is the following, due to Sabourand:

Agar-agar,	1.30;
Peptones,	.50;
Maltose,	3.80;
Water,	100.

Instead of test-tubes, Ellenmeyer flasks are used, so as to get a large flat surface for the growth to spread over from the point of inoculation in the center. The most favorable temperature for growth is 30° C.

Museum Preparations.—Specimens intended to be preserved for the museum should generally be gotten into pretty good shape by trimming and dissecting before they are placed in the hardening reagent. Of the liver or other large organs and tumors sections several cm. thick are generally preferable to the whole specimen. The usual custom in the past has been to wash the specimen for a number of hours or over night in running water, to get rid of the blood, and then to preserve in 80 per cent. alcohol. This method preserves form and relations well, but is nearly valueless for preserving colors.

Since the introduction of formaldehyde, from which at first much was expected in the way of faithful fixation of the normal colors of gross preparations, numerous attempts have been made to improve on the results obtainable with formaldehyde alone. Of the methods advocated, the following from Virchow's laboratory seems the most promising, and can be highly recommended:

Kaiserling's Method of Preserving the Natural Colors in Museum Preparations.—1. Fixation for one to five days in—

Formaldehyde,	200 c. c.;
Water,	1000 "
Nitrate of potassium,	15 grams;
Acetate of potassium,	30 "

Change the position of the specimen frequently, using rubber gloves to protect the hands from the injurious effect of the formaldehyde. The time of fixation varies with the tissue or organ and size of the specimen.

2. Drain and place in 80 per cent. alcohol one to six hours, and then in 95 per cent. alcohol for one to two hours, to restore the color, which is somewhat affected in the fixing solution.

3. Preserve in—

Acetate of potassium,	200 grams;
Glycerin,	400 c.c.
Water,	2000 "

Exposure to light gradually affects the colors. The process of fixation should be performed in the dark, and the specimens when preserved should be kept in the dark except when on exhibition.

If it seem desirable to cut a thin slice from the face of a specimen, this should not be done until the preparation has been in the preservative fluid two weeks. The specimen may then be placed in alcohol for one to two hours to brighten up the colors.

PATHOLOGICAL PRODUCTS.

Cloudy Swelling; Albuminous Degeneration.— The increase in the relative number of the albuminous granules of the protoplasm of the various tissue-cells in pathological processes is usually determined by examination of the fresh material, either macroscopically from the appearances on section, or microscopically from teased preparations or frozen sections mounted in salt solution. The organ as a whole (and therefore the individual cells) usually shows some increase in size. The nucleus is generally more or less obscured if the process is at all marked. According to Israel, the cloudiness must be recognizable with low powers and in places where the cells are massed together. The diagnosis should not be based on the appearances of single cells.

The chemical properties of the albuminous granules are the following: they disappear on treatment with dilute acetic acid (1–2 per cent. solution usually); they are not dissolved by chemical substances which dissolve fat (absolute alcohol, ether, chloroform, etc.); and they do not stain with osmic acid. The acetic-acid test is the one usually employed.

Albuminous degeneration can also be studied in sections of tissues hardened in certain of the fixatives and stained with diffuse colors. For this purpose hardening in Zenker's fluid and staining in alum-hematoxylin and eosin can be highly recommended.

Fatty Degeneration. — Fatty degeneration can be studied both in fresh and in properly hardened tissues. The recognition of fat in fresh specimens depends, aside from its physical peculiarities, on the following chemical properties:

1. It does not disappear on the addition of acetic acid.
2. It resists the action of dilute caustic potash or soda.
3. It is blackened by osmic acid.
4. It is dissolved (after dehydration in alcohol) by chloroform, ether, etc.
5. It stains red with sudan III.

The tests usually employed with teased preparations or frozen sections of fresh tissues are the reactions with acetic acid, sudan III, and osmic acid. The acetic acid is generally employed in a 1 or 2 per cent. solution, of which a few drops are placed at the edge of the cover-slip and drawn under by means of a bit of filter-paper placed at the opposite edge. The osmic acid in a 1 per cent. solution may be used in the same manner for teased preparations, or the tissue may be teased apart in a small quantity of the fluid. Frozen sections can be placed directly in it.

Since its introduction by Daddi in 1897, sudan III has largely replaced osmic acid for the study of fatty degeneration. The method is simpler, cheaper, and more reliable.

To stain fat with sudan III: 1. Place frozen sections for a few moments only in 95 per cent. alcohol.

2. Stain for a few minutes to twenty-four hours in a saturated solution of sudan III in 80 per cent. alcohol.

3. Wash off very quickly in 80 per cent. alcohol.

4. Wash in water.

5. Mount in water or glycerin.

Sections cannot be mounted in balsam. If sections are transferred directly from water to the stain, or directly back to water, more or less of a precipitate may be formed.

Fat is unaffected by formaldehyde or by solutions of the chrome salts, so that tissues preserved in them may be examined like fresh tissues in teased preparations or frozen sections so long as they remain in those fluids. This no longer holds true, however, after the tissues have once been transferred to alcohol.

The examination of fat in hardened tissues by means of osmium depends on its property of reducing osmic acid and thereby being stained black. Although osmic acid may be used alone for hardening tissues and staining fat, it is generally employed in combination with certain other fixatives. The two solutions generally selected are Flemming's and Marchi's.

Flemming's solution should be allowed to act from two to four days if the tissue is from 2–3 mm. thick, and then the pieces of tissue should be thoroughly washed in running water for twenty-four hours before being placed in alcohol.

Marchi's method was intended for differentiating fat from myelin (see page 251), but the solution employed by him may be used for staining fat in ordinary tissues. Place small pieces of tissue in it for five to eight days, wash thoroughly in running water, and harden in alcohol.

Marchi's method, carried through in the manner just described, succeeds perfectly with tissues fixed in formaldehyde.

Fat stained with osmium is soluble in ether, turpentine, xylol, and toluol, but is not dissolved by alcohol, chloroform, or oil of cloves. Imbedding in celloidin is not contraindicated, as the alcohol probably protects the fat from the injurious action of the ether. For the paraffin method clear in chloroform, and mount in properly prepared chloroform balsam.

Cholesterin crystals are recognized by their shape. On the addition of concentrated sulphuric acid the crystals turn yellow, and then rose-color. Treated with a little iodin, followed by concentrated sulphuric acid, they become colored violet, changing gradually to blue, green, and red.

Necrosis.—Necrosis in tissues is generally recognized by two features: either by the disappearance of the nuclei, although the cell-outlines may be visible, so that the nuclear stain is no longer possible, or by the presence of irregular, larger or smaller masses, generally supposed to be due to a fragmentation or breaking up of the chromatin, which stain intensely with nuclear stains. The disappearance of the nucleus is not synchronous with the death of the cell, but begins some twenty-four hours later, so that it is really evidence of changes following necrosis. It follows from the above that the microscopic evidence of necrosis is best studied in sections of tissues hardened in fixatives which favor nuclear staining, such as alcohol, Zenker's fluid, etc. Teased preparations and frozen sections of fresh tissue are much less useful.

For the study of sections from hardened material double stains with alum-hematoxylin and eosin, or, still better, with eosin followed by Unna's alkaline methylene-blue solution, are very useful, for the reason that the necrotic areas usually stain rather deeply with the diffuse stain, and are thereby brought out sharply.

For rendering the fragmented nuclei prominent the same methods may be followed as for mitosis. A fuchsin stain washed out by picric acid in the alcohol will often give excellent results.

Caseation is probably a tissue-change following local necrosis. Macroscopically and microscopically it resembles harder or softer cheese. Under the microscope it appears as coarsely or finely granular masses which have more or less completely lost the original tissue-structure. The chemical changes which have taken place have not been studied. Fibrin is rarely present. Caseous tissue possesses no peculiar staining reactions. Fragmented nuclei are frequently present in it, especially in the peripheries of the areas.

Demonstration of Fibrin.—Fibrin usually appears as delicate, transparent, slightly refractive threads which are often closely matted together so as to form large masses. More rarely it appears as coherent masses of the finest granules, as homogeneous glassy lumps, or as thin sheets. The characteristic reaction for fresh fibrin is that it quickly swells up and optically dissolves in very dilute acetic acid.

Fibrin is well brought out in sections of hardened tissues by a double stain of alum-hematoxylin and eosin, or of eosin followed by Unna's alkaline methylene-blue solution, especially if the specimens have been fixed in Zenker's fluid.

Weigert's Differential Stain for Fibrin.

1. Harden in alcohol. Good results can also usually be obtained after Zenker's fluid.
2. Stain sections in lithium carmine.
3. Stain in aniline-gentian-violet five to twenty minutes.
4. Wash off with normal salt solution.
5. Iodin solution 1 : 2 : 100 one minute.
6. Wash off with water.
7. Decolorize in—

Aniline,	2 parts;
Xylol,	1 part.

8. Wash off with three changes of xylol.
9. Xylol balsam.

The fibrin and those bacteria which are stained by Gram are stained blue. The nuclei are red if the decolorization is carried far enough. It can easily be watched under the low power of the microscope. The method is not always successful, especially with tissues which are old. It is sometimes advisable to increase the proportion of xylol in the decolorizing solution, so that the extraction of the color may not be so rapid. Besides the fibrin, certain forms of hyaline are often stained by this method. Fibrin can be stained by the above procedure in sections of tissues hardened in Müller's fluid if the specimens are first placed for several hours in a 5 per cent. aqueous solution of oxalic acid to reduce the bichromate of potassium.

Differential stains for fibrin are also obtained by the chlorid of iron hematoxylin stain (page 293), and by the connective-tissue stain (page 303). The former is applicable after any fixing reagent, the latter only after corrosive sublimate and Zenker's fluid.

Mucin.—The term "mucin" is applied to a proteid substance having certain chemical reactions, and also to certain other substances which give the same reactions, but do not belong to the proteids. These various substances of secretory and degenerative origin cannot be distinguished microscopically, and have been investigated but little chemically. The reactions in common are the following: they dissolve in water to form a slimy fluid; they are precipitated from slightly alkaline solutions by acetic acid; the fresh precipitate dissolves in alkalies and in neutral salt solutions. Acetic acid, usually employed for this purpose in a 1 or 2 per cent. solution, precipitates mucin in the form of threads or granules. This reaction with fresh tissues has long been the main test for mucin. The acetic acid is drawn under the cover-slip by means of filter-paper placed at the opposite edge. The preparation should be mounted in water, not in salt solution, which may hinder or entirely prevent the reaction from taking place. Of late certain color reactions have become prominent. Mucin is coagulated into threads by alcohol or corrosive sublimate, and in this form can be stained by a number of staining reagents. Alum-hematoxylin under certain conditions will stain mucin. According to P. Mayer, these conditions depend on a certain degree of ripeness of the solution, on the presence of enough alum to keep the nuclei from staining deeply, and, most important of all, on the absence of any free acid. This is difficult to manage, unless the solution is carefully neutralized, on account of the acid properties of alum. Mayer therefore recommends staining the sections in muchematein (see page 267).

Various aniline dyes have been recommended for staining mucin: those most favorably spoken of are methylene-blue (Orth), Bismarck brown (P. Mayer), thionin (Hoyer), polychrome methylene-blue (Unna), and toluidin-blue. The drawback to most of the aniline stains is that they are quickly extracted by the alcohol used for dehydrating. On this account P. Mayer highly recommends Bismarck brown, because permanent mounts can be easily made with it. It is not extracted by alcohol, and it does not fade in Canada balsam like many of the others.

Hardening in corrosive sublimate and imbedding in paraffin are generally recommended as preferable to hardening in alcohol and imbedding in celloidin. Stain sections for five to fifteen minutes in a rather dilute aqueous solution of the dye chosen. Of Bismarck brown use a saturated aqueous solution, and stain, if necessary, twenty-four hours. With thionin, toluidin-blue, and polychrome methylene-blue metachromatic stains are obtained; the mucin is colored red, the rest of the tissue blue. Two special methods for staining mucin are given in detail:

Hoyer's Method with Thionin.—Mucin, red; everything else, blue. 1. Harden in corrosive sublimate, followed by alcohol.

2. Paraffin sections are passed through xylol, chloroform, and 95 per cent. alcohol to free them from paraffin, and are

then placed in a 5 per cent. aqueous solution of corrosive sublimate for three to five minutes.

3. Stain in a dilute solution of thionin for ten to fifteen minutes.
4. Alcohol.
5. Clear in the mixture of the oils of cloves and thyme.
6. Turpentine oil or oil of cedar.
7. Balsam.

Before the staining the sections must not be treated with iodin solution to get rid of the precipitate of mercury, because it spoils the staining.

Unna's Method with Polychrome Methylene-blue.—1. Stain paraffin or celloidin sections hardened in alcohol in polychrome methylene-blue five to ten minutes or longer.

2. Wash in acidulated water.
3. Fix in 10 per cent. solution of bichromate of potassium half a minute.
4. Wash in water.
5. Dry on slide with filter-paper.
6. Decolorize in aniline plus 1 per cent. hydrochloric acid (a few seconds only).
7. Wash off with oil of bergamot.
8. Balsam.

Pseudo-mucin dissolves in water to form a slimy material, and is precipitated from its solutions by alcohol in thread-like masses which are again soluble in water. It is not affected by acetic acid. Pseudo-mucin is found in certain ovarian and other tumors.

Colloid and Hyaline.—The terms colloid and hyaline are not yet sharply limited to definite chemical substances. The term colloid was originally applied to the homogeneous substance found in the thyroid gland, but has been broadened to include various substances of a similar appearance. The term hyaline is still more indefinite, but its use may be said to be applied most generally to those homogeneous substances which stain deeply with various stains, in contradistinction to those which like colloid show no marked affinity for staining reagents after ordinary fixatives.

Unquestionably, numerous substances of different chemical composition and of varying origin have been grouped under these two titles because of their physical and optical characteristics—namely, that they occur as glassy, refractive, homogeneous, occasionally colored gelatinous or firm masses. Chemically, very little that is definite is known about them, and they possess no peculiar chemical reactions. Several attempts have been made to classify them in accordance with their reactions to various staining reagents.

Von Recklinghausen applied the term colloid to all the homogeneous, transparent-looking substances, including mucin, amyloid, etc., and reserved the term hyaline for a special group, which, according to him, is characterized by the following peculiarities : it resembles amyloid in physical characteristics, but does not react to iodin ; it stains deeply with acid dyes, such as eosin and acid fuchsin.

Ernst has recently endeavored to differentiate two groups of hyaline substances, colloid and hyaline, by means of their reaction to Van Gieson's picro-acid fuchsin solution. According to him, true hyaline stains with acid fuchsin alone, and appears of a deep-red color, while colloid, of which the typical example is found in the thyroid gland, stains with both picric acid and acid fuchsin, so that it appears of an orange or yellowish-brown color. He has also tried to prove that all colloid is derived from epithelial cells, while all hyaline comes from connective tissue or from blood-vessels.

According to Von Kahlden, these differential staining reactions with Van Gieson's mixture claimed by Ernst for colloid and hyaline are by no means justifiable, because true colloid often stains a deep red. Furthermore, Unna has shown that in the skin connective-tissue cells can give rise to the so-called true hyaline, of which part is acidophilic and part basophilic, while the intercellular substance gives rise to colloid.

The last attempt to classify the various homogeneous substances on the basis of their reactions to dyes, apparently the only method possible at present, has been made by Pianese as a result of his studies of the various degenerative processes occurring in cancer-cells. He used a special fixative (see p. 249) and five different staining methods (see p. 275, methods III. A and B, IV., V., and VI.). Of these methods, III. B is the best, because it gives a characteristic color to each substance—hyaline, brick-red; colloid, bright green; mucin, clear sky-blue; and a substance resembling amyloid, a dark reddish-violet. Besides these distinct reactions for colloid, hyaline, mucin, and a substance resembling amyloid, he found others less definite; one of these he calls pseudo-mucin and another pseudo-colloid. As a basis for his studies he took the reactions of amyloid, mucin (intestine), colloid (thyroid gland), and hyaline (hyaline remains of ovarian follicles, hyaline degeneration of renal glomeruli), with the same stains after fixation in his own hardening mixture.

The above brief historical statement is considered necessary to show the present views in regard to these various, more or less indefinite, homogeneous, transparent substances. For demonstrating them after the usual hardening reagents, of which alcohol and corrosive sublimate are perhaps the best, a double stain with alum-hematoxylin and eosin is very useful. Certain of the homogeneous substances stain deeply with eosin; others, like the transparent drops and masses occasionally found in the walls of the blood-vessels of the brain, stain with hematoxylin. Sometimes good results can be obtained with Weigert's fibrin stain or with carbol-fuchsin. The most generally useful stain, aside from alum-hematoxylin and eosin, is probably Van Gieson's mixture.

1. Stain deeply in alum-hematoxylin.
2. Wash in water.
3. Stain three to five minutes in a saturated aqueous solution of picric acid, to which is added enough of a saturated aqueous solution of acid fuchsin to give it a deep-red color. The effect of various proportions is sometimes useful.
4. Wash in water.
5. Alcohol.
6. Oleum origani cretici.
7. Balsam.

The transparent homogeneous substances usually stain from orange to deep red in color; connective tissue, red.

Unna's Method for Hyaline and Colloid Material.—*A*. Harden in alcohol. 1. Acid fuchsin (2 per cent. aqueous solution) five minutes.

2. Saturated aqueous solution of picric acid two minutes.
3. Saturated alcoholic solution of picric acid two minutes.
4. Wash off in alcohol.
5. Oil, balsam.

Hyaline and connective-tissue fibers, red; colloid of thyroid gland, yellow; protoplasm, yellow.

B. To show acidophilic and basophilic hyaline: 1. Water-blue (2 per cent. aqueous solution) twenty to thirty seconds.

2. Water.
3. Carbol-fuchsin one to two minutes.
4. Water.
5. Alcohol slightly tinged with iodin.
6. Pure alcohol.
7. Oil, balsam.

Nuclei, keratin, and large hyaline masses, cherry red; connective-tissue fibrillæ, protoplasm, and small hyaline bodies, blue.

For finer work the methods of Pianese should be used.

Keratohyalin (*Unna*).—1. Stain sections in a fairly old alum-hematoxylin solution until they are over-stained.

2. Place in a very weak solution of permanganate of potassium (about 1 : 2000) for ten seconds.
3. Dehydrate and decolorize in alcohol.
4. Oil, balsam.

An isolated stain of the granules of keratohyalin is obtained, blue-black in color.

In like manner a 33 per cent. solution of sulphate of iron acting for ten minutes, or a 10 per cent. solution of chlorid of iron for a few seconds, will produce the same effect. Ordinarily, sections are stained deeply in alum-hematoxylin, and decolorized with acetic acid and alcohol or with hydrochloric acid and alcohol.

Glycogen Infiltration.—Glycogen is a carbohydrate of slightly varying composition, occurring in cells, more rarely in the intercellular tissue, either diffusely or more commonly in the form of larger and smaller masses and granules of a transparent homogeneous appearance. It is demonstrated micro-chemically by means of its reaction with iodin, which stains it brown. It is easily differentiated from amyloid by the fact that with the exception of the glycogen from certain sources, such as cartilage-cells, it is readily soluble in water and does not give the iodin-sulphuric-acid reaction.

In consequence of its property of dissolving readily in water the aqueous Lugol's solution of iodin cannot be employed for staining glycogen in fresh tissues. Instead, a thick solution of gum arabic containing 1 per cent. of Lugol's solution must be used, or, better still, equal parts of glycerin and Lugol's solution, in which the sections are more perfectly cleared.

For sections hardened in absolute alcohol the same methods may be used, but better results, and practically permanent mounts, can be obtained by the method of Langhans. Lugol's solution is used for staining the sections, because after hardening in alcohol the glycogen is much less soluble in water than in the fresh state. The iodin-glycerin mixture would probably be better.

1. Stain in Lugol's solution.
2. Dehydrate in 1 part of tincture of iodin to 3 or 4 parts of absolute alcohol.
3. Clear in oleum origani cretici.

The sections are to be preserved in oil. Even a ring of balsam around the cover-slip will cause the color to fade. Other oils are not so good.

Lubarsch recommends the following method: 1. Fixation in absolute alcohol.

2. Stain for five minutes in the following solution, which should be filtered and carefully protected from sunlight:

Delafield's hematoxylin,	2;
Lugol's solution,	2;
Water,	1.

3. Alcohol, xylol, xylol balsam. Glycogen brown, nuclei blue.

Amyloid Infiltration.—Amyloid is insoluble in water, alcohol, ether, and dilute acids, and is not digested by pepsin and hydrochloric acid. It is distinguished from the other homogeneous substances, except glycogen, by the fact that it is stained mahogany-brown by iodin in solution. If a section containing amyloid be quickly and lightly stained in iodin solution and then transferred to sulphuric acid, the color of the amyloid will usually change at once or in a few minutes from red, through violet, to blue. Sometimes the color turns simply of a deeper brown. Several of the aniline dyes give almost as perfect characteristic color-reactions for amyloid as iodin, and are perhaps better for the purposes of histological study. Any of these differential stains may be used with fresh or hardened tissues. Alcohol as a hardening reagent gives the best results, but the other fixatives may be employed. Unfortunately, good permanent mounts cannot be made with any of the characteristic stains, so that the ordinary double stains of alum-hematoxylin with eosin or Van Gieson's mixture will often be found of the greatest help in studying the distribution of amyloid. The aniline-blue connective-tissue stain can also be highly recommended because it stains amyloid light blue, so that it stands out in marked contrast to the red of the liver-cells.

Iodin Reaction for Amyloid.—1. Stain sections in a weak solution of iodin (Lugol's solution diluted until of a clear yellow color) for three minutes.

2. Wash in water.

3. Mount and examine in water or glycerin.

If the tissue reacts strongly alkaline, a condition which may result from post-mortem decomposition, the color reaction with iodin will not take place. In such cases the tissue or the sections of it should be treated with dilute acetic acid before applying the test. The normal reaction of amyloid with iodin may be increased by treating the section after staining with dilute acetic acid.

Langhans' Method for Obtaining Permanent Mounts with Iodin.—1. Harden in alcohol.

2. Stain sections in Lugol's solution.

3. Dehyhrate in 1 part of tincture of iodin to 3 or 4 parts of absolute alcohol.

4. Clear and mount in oleum origani cretici.

The color is said to keep remarkably well. Other oils or balsam cause it to fade quickly. The staining in Lugol's solution may be omitted, as the tincture of iodin usually stains the amyloid sufficiently deeply.

Iodin and Sulphuric-acid Reaction.—1. Stain quickly and lightly in dilute Lugol's solution.

2. Treat with sulphuric acid, either concentrated or dilute (1 to 5 per cent.), on the slide or in the staining dish. Strong hydrochloric acid may be used in the same way.

The change of colors from red to blue already spoken of usually occurs within a few minutes, but occasionally does not take place at all.

The following substances give reactions with the above iodin tests:

1. *Cholesterin crystals* are stained rather dark with dilute iodin solution, and turn a beautiful blue color at the edges on the addition of strong sulphuric acid.

2. The *corpora amylacea* in the prostate and central nervous system stain brown with the dilute iodin solution.

3. *Starch-granules* stain blue with dilute iodin solution.

4. *Cellulose* stains yellow with iodin. If washed and treated with strong sulphuric acid, it turns blue where the acid touchs it.

For the reactions with the aniline dyes the sections must be free from celloidin.

Reaction with Methyl- or Gentian-violet.—1. Stain in 1 per cent. methyl-violet three to five minutes.

2. Wash in water plus 1 per cent. of hydrochloric acid.

3. Examine in water or in glycerin.

The stain will keep for some time if mounted in a saturated solution of acetate of potash or in levulose. Other methods are to stain in aniline-gentian-violet and to wash out in a 1 or 2 per cent. solution of acetic acid, or to stain in a strong solution of methyl-violet to which acetic acid is added, and to wash out in water. The amyloid is stained violet-red, the tissue blue.

Reaction with Iodin-green.—1. Stain fresh or hardened sections in a $\frac{1}{3}$ per cent. aqueous solution of iodin-green for twenty-four hours.

2. Wash in water.

3. Mount in water or glycerin.

Amyloid, a violet-red; tissue, green. Stilling claims that the reaction is surer than with methyl-violet.

Reaction with Bismarck Brown and Gentian-violet (Birch-Hirschfeld).—1. Stain in a 2 per cent. alcoholic solution of Bismarck brown five minutes.

2. Wash in absolute alcohol.

3. Wash in distilled water ten minutes.

4. Stain in a 2 per cent. solution of gentian-violet five to ten minutes.

5. Wash in dilute acetic-acid solution.

6. Mount in levulose.

Amyloid, red; tissue, brown.

Pigmentation.—The various pigments found in the human body under normal and pathological conditions may be divided into three groups:

1. **Hematogenous pigments,** derived from the coloring matter of the blood.

(*a*) *Hemoglobin* and *methemoglobin:* soluble in water and alcohol not absolute; occur as yellowish to yellowish-brown granules and droplets; stain deeply with eosin after proper fixation; occur in hemoglobinuria, etc.

(*b*) *Parhemoglobin:* a form of hemoglobin; crystallizes like it, but is insoluble in alcohol.

(*c*) *Hematoidin=bilirubin:* contains no iron; is insoluble in water, alcohol, and ether; dissolves in chloroform; occurs as yellow or brown amorphous material or as crystalline rhombic plates and needles. Is found in extravasations of blood.

(*d*) *Hemosiderin:* occurs as bright-colored, yellowish-brown and brown granules and masses; gives iron reaction; is insoluble in water, alcohol, and ether; is found in extravasations of blood, in the liver in pernicious anemia, etc.

(*e*) *Melanin:* occurs as dark-brown or black granules and masses; does not give iron reaction; is found in malaria in the red blood-globules and in the tissues of the spleen, liver, and brain, but not of the lungs.

(*f*) *Bile-pigment=bilirubin=hematoidin:* insoluble in water, ether, and alcohol; occurs as yellowish granules and masses which are often greenish if old; is found in jaundice.

2. **Autochthonous pigments,** formed by cells from colorless elements of nutrition. They all occur microscopically as lighter or darker brown granules; are insoluble in water, alcohol, dilute caustic potash, etc., and contain no iron. They are found in the iris, retina, skin, ganglion-cells, Addison's disease, melanotic sarcomata, etc.

3. **Extraneous pigments,** entering the body from without. The most common examples are carbon in anthracosis pulmonum, iron in siderosis pulmonum, silver in argyria.

Pigments are recognized microscopically, partly by their color and form, partly by their chemical reactions, and partly, though less accurately, by the lesions or pathological processes in connection with which they occur. They usually show best in contrast to red nuclear stains, such as alum or lithium carmine, but alum-hematoxylin often gives excellent results.

The pigments of the second and third groups are perfectly preserved by all of the ordinary fixatives, of which alcohol, corrosive sublimate, and Zenker's fluid can be particularly recommended. Of the first group, melanin and hematoidin are preserved in any fixative. Hemoglobin and methemoglobin must be fixed in the solutions recommended for red blood-globules—namely, Zenker's fluid, corrosive sublimate, and Müller's fluid. Parhemoglobin and hemosiderin should be preserved in alcohol. Bile-pigment is turned green, according to Ziegler, by fixation in corrosive sublimate, and is thereby rendered more prominent. In alcohol it preserves its yellow color.

Carbon may be distinguished from melanin or any of the other pigments by the fact that it is insoluble in concentrated sulphuric acid. The only pigment for which micro-chemical color reactions are generally employed is hemosiderin, which really represents a group of pigments containing iron instead of one definite compound. Most of them will show the iron reaction after a shorter or longer time, but others, like the hemoglobin from which they are all derived, refuse to give it.

The iron compounds present are usually ferric salts, but occasionally ferrous. Both groups react in the same way to sulphate of ammonium, but only the ferric salts react to ferrocyanide of potassium. For the ferrous salts, which occur much more rarely, the ferricyanide of potassium must be used. Dr. E. S. Wood suggests that a mixture of ferro- and ferricyanide of potassium be employed, so as to demonstrate at once both groups of iron compounds, as with the sulphate-of-ammonium method. In performing the iron reactions steel needles must be avoided.

Reactions for Iron in Hemosiderin.—Tissues should be hardened in alcohol. I. Reaction for ferric salts with ferrocyanide of potassium and hydrochloric acid.

A. 1. Place sections for five to twenty minutes or longer in a 2 per cent. aqueous solution of ferrocyanide of potassium.

2. Transfer to acid alcohol (HCl 1 c.c. to 70 per cent. alcohol 100 c.c.) for five to ten minutes, or to glycerin plus $\frac{1}{2}$ per cent. hydrochloric acid. The iron appears bright blue in color.

If desired, the sections can be washed out after the acid alcohol, and passed through alcohol and oil to balsam. After the iron reaction has been performed the nuclei may be stained in either alum or lithium carmine, or a little ferrocyanide of potassium may be added to the lithium carmine, and the reaction and nuclear stain thus effected by the same step.

B. H. Stieda's Method for Permanent Mounts with Nuclear Stains.—1. Stain several hours in lithium carmine.

2. Wash off quickly in water.

3. Place from four to six hours in a 2 per cent. aqueous solution of ferrocyanide of potassium.

4. Transfer to acid alcohol for six to twelve hours.

5. Wash quickly in water.

6. Alcohol, oil, Canada balsam.

II. The reaction for the ferrous salts is performed in the same way as for ferric salts, with the exception that ferricyanide of potassium is used instead of the ferro- compound.

III. *Reactions for Ferric and Ferrous Salts.*—*A.* Use a mixture of ferro- and ferricyanide of potassium (1 gram each to 100 c.c. of water), followed by acid alcohol. Nuclear stains and permanent mounts may be made as above.

B. 1. Place sections in a freshly prepared solution of sulphate of ammonium for five to twenty minutes, until they are dark or black-green in color.

2. Wash quickly in water.

3. Examine in glycerin or pass through alcohol and oil to Canada balsam.

The iron appears in the form of black or dark-greenish granules. Sulphate of ammonium causes similar precipitates with other metal salts, such as the nitrates of silver, lead, and mercury.

Petrifaction.—Calcification, the more common form of petrifaction, is the term applied to the infiltration of tissues with phosphate and carbonate of lime. The salts appear microscopically as small, very refractive granules which may be mistaken for fat, or as large masses due to the fusion of granules. They are dissolved by hydrochloric or nitric acid (5 per cent. solution). If carbonate of lime is present, bubbles of carbon-dioxid are set free. Phosphate of lime dissolves without effervescence. To differentiate between lime-salts and other substances soluble in hydrochloric acid use concentrated sulphuric acid to form sulphate of lime (gypsum), which appears as fine, short, radiating needles. On dissolving out the lime-salts a matrix of dead tissue or of hyaline material will usually be found left behind. As a rule, this hyaline material stains deep blue in alum-hematoxylin or red in Van Gieson's mixture.

Another form of petrifaction is that found in gout, due to the infiltration of certain tissues with uric-acid salts, of which urate of sodium is the most common. The crystals are soluble with difficulty in cold water, insoluble in alcohol and ether.

THE STAINING OF BACTERIA IN TISSUES.

Bacteria are demonstrated in sections of tissues almost entirely by means of the aniline dyes, of which three have thus far proven themselves to be particularly valuable—namely, methylene-blue, gentian-violet, and fuchsin. These dyes are employed in aqueous or dilute alcoholic solutions, of which the effective staining power is greatly increased by means of heat and by the addition to the solutions of certain chemical substances.

The effect of moderate heat is obtained by placing the sections in the incubator for several hours, or greater heat for a short time is utilized by warming the staining solution on the slide over a small flame for a few seconds or minutes, keeping the fluid steaming, but not allowing it to boil.

Of the various methods employed to increase the staining power of aniline dyes by means of chemical substances, the most successful have been the use of caustic potash with methylene-blue, of aniline oil with gentian-violet and fuchsin, and of carbolic acid with fuchsin and methylene-blue.

For decolorizing sections after they have been stained the most commonly employed reagents are—

1. Acetic acid in dilute aqueous solutions 1 : 100, 1 : 1000.
2. Alcohol.
3. Iodin in iodid-of-potash solution (with certain dyes only).
4. Mineral acids in various strengths.
5. Chlorid of aniline.
6. Acid aniline colors added to the alcohol to increase its extractive power.
7. Aniline and ethereal oils.

The choice of a decolorizer varies with the staining solution employed and with the organism that is to be stained.

Sections which are to be stained for bacteria may be divided into two classes:

1. Sections free from celloidin, subdivided into—
    - *a.* Sections cut without an imbedding mass;
    - *b.* Paraffin sections;
    - *c.* Sections from which the celloidin has been removed.
2. Sections infiltrated with celloidin.

Celloidin imbedding is to some extent a drawback to the stains for certain organisms, because the celloidin tends to hold the color, so that the bacteria are not so distinct as they otherwise would be. Still, it is so important to be able to stain bacteria in celloidin sections that particular care is devoted in the following pages to methods which obviate most of the difficulties.

Paraffin sections should, as a rule, be attached to the slide by means of Mayer's glycerin-albumin mixture.

It will usually be found advisable to attach celloidin sections to the slide by means of ether-vapor. They will then keep perfectly flat in any staining solution, and may be heated without danger of wrinkling or contracting. The heat should never be applied directly under a section, but at one end of the slide.

All bacteria yet known will stain when placed in appropriate staining solutions. Some, however, are stained quickly, while others are stained with difficulty; some give up the stain readily to decolorizers, while others retain it tenaciously. In consequence of their reactions to certain dyes and to certain decolorizers, bacteria, from the point of view of staining, may be divided into three groups:

1. Bacteria which do not stain by Gram;
2. Bacteria which stain by Gram;
3. Bacteria which stain by the tubercle bacillus method.

Two at least of the organisms in the third group will also stain by Gram. The organisms of the second and third groups are much more easily demonstrated in tissues than those in the first group, because it is possible to stain them of one color and the nuclei of the cells of another color. In other words, it is possible to stain them so that they are differentiated from the tissue in which they lie, and hence stand out prominently.

The organisms of the first group have no differential stain; they take the same color as the nuclei of the tissue. Moreover, although they stain easily, most of them do not stain deeply, and readily part with the color they have taken up.

Pathogenic Bacteria which do not Stain by Gram.

(See also page 94).

Gonococcus;
Diplococcus intracellularis meningitidis;
Typhoid bacillus;
Bacillus coli communis;
Glanders bacillus;
Bacillus of malignant edema;
Influenza bacillus;
Bacillus pyocyaneus;
Bacillus mucosus capsulatus;
Colon bacillus;
Spirillum of Asiatic cholera.

Of these organisms certain ones deserve special mention on account of their frequent occurrence or on account of the difficulty of demonstrating them in tissues, and certain variations in staining methods which have proved serviceable will be given. Löffler's methylene-blue solution is generally considered the most useful stain for this class of bacteria, but excellent results can also be obtained with Unna's alkaline methylene blue solution preceded by eosin, especially after fixation in Zenker's fluid.

Löffler's Methylene-blue Solution.—1. Stain paraffin sections twenty minutes to twenty-four hours.

2. Wash in weak acetic acid, 1 : 1000, for ten to twenty seconds.

3. Absolute alcohol, two or three changes, to differentiate and dehydrate (as a rule, only a few seconds are required for this step).

4. Xylol.

5. Xylol balsam.

For celloidin in sections use 95 per cent. alcohol; blot, and treat with xylol; repeat until sections are clear; mount in xylol balsam.

This solution of methylene-blue is extremely useful, because it will stain all bacteria except the tubercle-bacillus group. Other solutions which may be used in the same way are — aniline-gentian-violet, Stirling's solution of gentian-violet, simple aqueous solutions of gentian- or methyl-violet, and Ziehl's carbol-fuchsin.

Gonococcus.—Löffler's solution gives good results.

Touton recommends staining sections in carbol-fuchsin and washing out in alcohol.

Typhoid Bacillus.—Typhoid bacilli in stained sections are generally best hunted for with a low power. The characteristic colonies which they form are easily recognized. Good results in staining can be obtained with Löffler's methylene-blue solution used in the manner already described, but the stain is never very intense. For rendering the bacilli rather more prominent, so that small groups of them may be recognized, Flexner has recently advised the two following methods:

A.—1. Stain paraffin sections in Löffler's methylene-blue solution for two hours.

2. Acetic-acid solution, 1 : 1000, for several minutes.
3. Dehydrate in absolute alcohol.
4. Oil of cloves to clear and differentiate.
5. Xylol, several changes.
6. Xylol balsam.

B.—1. Stain sections in Stirling's gentian-violet solution for ten minutes.

2. Acetic-acid solution, 1 : 1000, for some minutes.
3. Dehydrate quickly in 95 per cent. alcohol.
4. Transfer to slide, blot, add oil of cloves to clear, and differentiate. Change the oil several times until the desired differentiation is obtained.
5. Wash off section several times with xylol.
6. Xylol balsam.

Influenza Bacillus.—1. Harden in alcohol.

2. Stain half an hour or more in carbol-fuchsin diluted with 20 parts of water.

3. Wash out in a watch-glass of water to which is added a drop of glacial acetic acid until the section appears gray-violet in color.

4. Alcohol, xylol, balsam.

Glanders Bacillus.—The bacilli are usually not numerous, and are scattered about in a mass of deeply-staining fragmented nuclei, so that they are recognized with great difficulty.

Löffler's Method for Sections.—1. Stain paraffin sections twenty minutes in Löffler's methylene-blue solution or in equal parts of aniline-gentian-violet and 1 : 10,000 KOH solution.

2. Place for five seconds in the following solution:

Distilled water,	10 c.c.;
Concentrated sulphuric acid,	2 drops;
5 per cent. oxalic acid,	1 drop.

3. Wash out quickly in distilled water.

4. Absolute alcohol.

5. Xylol.

6. Xylol balsam.

It is recommended to place the section for a few minutes before staining in the 1 : 10,000 caustic-potash solution.

Schütz's Method.—1. Stain twenty-four hours in equal parts of concentrated alcoholic solution of methylene-blue and caustic potash, 1 : 10,000.

2. Wash in acidified water.

3. 50 per cent. alcohol for five minutes.

4. Absolute alcohol for five minutes.

5. Xylol.

6. Canada balsam.

Noniewicz's Method.—1. Stain in Löffler's methylene-blue solution two to five minutes.

2. Wash in water.

3. Decolorize one to five seconds in

½ per cent. acetic acid,	75 parts;
½ per cent. aqueous solution of tropeolin,	25 "

4. Wash in water.

5. Dehydrate section on slide with filter-paper; then in the air; finally, over small flame.

6. Clear by dropping xylol on it repeatedly.

7. Xylol balsam.

Friedländer's Capsule-bacillus.—The following method is recommended for staining the capsules in sections:

1. Stain for twenty-four hours in the incubator in the following solution:

Concentrated alcoholic solution of gentian-violet,	50;
Distilled water,	100;
Glacial acetic acid,	10.

2. Wash out in a 1 per cent. solution of acetic acid.

3. Alcohol.

4. Oil.

5. Canada balsam.

If the process of decolorization is stopped at the right moment, the capsules will be pale blue, while the bacilli will be stained deep blue.

Pathogenic Bacteria which Stain by Gram.

Micrococcus lanceolatus;
Streptococcus pyogenes;
Staphylococcus pyogenes aureus, albus, citreus, and flavus;
Micrococcus tetragenus;
Anthrax bacillus;
Bacillus of rhinoscleroma;
Diphtheria bacillus;
Tetanus;
Bacillus aërogenes capsulatus;
Actinomyces;
Tubercle bacillus; }
Leprosy bacillus. }

These organisms, with the exception of the tubercle-bacillus group, are all readily stained by the general methods employed for staining under Group 1. For staining most of them in sections, however, the differential Gram-Weigert method will be found to give the most satisfactory results.

The Gram Staining Method.—Directions for staining paraffin sections: 1. Stain in aniline-gentian-violet five to twenty minutes.

2. Wash in normal salt solution or water.
3. Iodin solution (1 : 2 : 300) one minute.
4. Wash in water.
5. Absolute alcohol, several changes, until no more color is given off and the section is apparently decolorized.
6. Xylol.
7. Xylol balsam.

This method is not suited for celloidin sections, because the alcohol does not decolorize the celloidin sufficiently. In fact, it is better to reserve Gram's method for cover-slip work alone, and to use instead of it, for sections of all kinds, Weigert's modification. This consists simply in the use of aniline oil instead of alcohol as a decolorizer. The method is easily acquired, is perfectly adapted to celloidin sections, and the results are more perfect than after Gram.

The Gram-Weigert Staining Method.—Directions for staining celloidin sections: 1. Stain sections with lithium carmine in the ordinary way (see page 269).

2. After dehydrating in 95 per cent. alcohol stick the section to the slide with ether-vapor.
3. Stain in aniline-gentian-violet five to twenty minutes.
4. Wash off excess of stain in normal salt solution.
5. Iodin solution (1 : 2 : 100) one minute.
6. Wash off in water.
7. Blot section with filter-paper to remove as much of the moisture as possible.
8. Aniline oil, several changes, to dehydrate and to remove all excess of color.
9. Xylol, several changes to remove the aniline oil completely.
10. Xylol balsam.

Bacillus of Rhinoscleroma.—Method of staining capsules in sections of tissues hardened in alcohol (Wolkowitsch): 1. Stain twenty-four to forty-eight hours in aniline-gentian-violet.

2. Wash off in water.
3. Iodin solution one to four minutes.
4. Absolute alcohol.
5. Oil of cloves, which removes still more of the color.
6. Xylol.
7. Canada balsam.

According to Wolkowitsch, the hyaline masses in rhinoscleroma stain intensely with methyl-violet, gentian-violet, methylene-blue, and fuchsin; less with safranin, and not at all with hematoxylin. Eosin stains them well. Double staining with hematoxylin and eosin is therefore to be recommended highly.

Actinomyces.—In staining the actinomyces it is important to stain not only the filaments and other forms of the organism, but also the hyaline swollen sheaths which surround the ends of the filaments. Eosin followed by methylene-blue sometimes gives good results. Good preparations can also be obtained by staining in alum hematoxylin, followed by a strong solution of eosin; place the sections for five to thirty seconds in acid alcohol, and then wash thoroughly in water before dehydrating in alcohol. It is believed that the two following methods will give better results than can be obtained by any of the methods previously published for this purpose. The first is, perhaps, the better and surer, although the clubs are sometimes brought out more intensely by the second method.

Method No. 1 (*Mallory*).—1. Stain sections deeply in a saturated aqueous solution of eosin for at least ten minutes.

2. Wash off in water.
3. Stain in aniline-gentian-violet two to five minutes.
4. Wash off with normal salt solution.
5. Iodin solution (1 : 2 : 100) one minute.
6. Water. Blot with filter-paper.
7. Aniline oil till section is clear.
8. Xylol, several changes.
9. Xylol balsam.

A light preliminary stain with alum-cochineal will often be found useful.

Method No. 2 (*Mallory*).—1. Stain lightly in alum-cochineal three to five minutes.

2. Wash in water.
3. Dehydrate in 95 per cent. alcohol.
4. Fasten section to slide with ether-vapor.
5. Aniline-gentian-violet five to twenty minutes.
6. Wash off with water.
7. Dry with filter-paper.
8. Aniline saturated with fuchsin one to three minutes.
9. Wash out the fuchsin with pure aniline until the clubs are sharply differentiated: watch the process under the low power of the microscope.
10. Xylol, several changes.
11. Xylol balsam.

The polymorphous bacterium is stained blue, the swollen membrane (the club), light to dark pink. Alum-cochineal furnishes a better contrast to the actinomyces than either alum-carmine or alum-hematoxylin. By these methods it is possible to demonstrate in sections containing young colonies the ends of the threads stained blue surrounded by the swollen cell-membrane stained pink.

Bacteria that Stain by the Tubercle Bacillus Method.

Tubercle bacillus;
Leprosy bacillus;
Smegma bacillus;
Syphilis bacillus.

The important point about staining *tubercle bacilli* is to stain them deeply enough in the beginning; then there is little danger of their fading in the subsequent steps of contrast-staining. It is probable that carbol-fuchsin, used hot, is the most powerful stain we have for this purpose. If the solution is steamed, generally on the slide, one to five minutes are probably sufficient for all purposes. Tubercle bacilli stain well, not only after alcohol, but also after most of the other fixing reagents, such as corrosive sublimate, Zenker's fluid, Flemming's solution, etc.

Ehrlich's Method.—1. Stain paraffin sections in aniline-fuchsin or gentian-violet for half an hour to twenty-four hours, or for one to five minutes if solution is heated to steaming.

2. Wash in water.

3. Decolorize in 20 per cent. nitric acid one-half to one minute.

4. Wash in 70 per cent. alcohol until no more color is given off.

5. Contrast-stain in a saturated aqueous solution of methylene-blue or of Bismarck brown one to two minutes.

6. Wash in water.

7. Dehydrate in absolute alcohol.

8. Xylol, xylol balsam.

Ziehl-Neelson-Gabbet Method.—1. Stain paraffin sections in carbol-fuchsin solution, warming the solution so that it steams one to three minutes.

2. Wash in water.

3. Decolorize and stain for contrast in sulphuric-acid-methylene-blue solution one minute (see page 271).

4. Wash in water.

5. Absolute alcohol.

6. Xylol.

7. Xylol balsam.

This method is not suited to celloidin sections, because the celloidin retains too deep a blue stain.

Kühne's Method.—1. Stain paraffin sections lightly in alum-hematoxylin.

2. Wash in water.

3. Stain in carbol-fuchsin one to five minutes if warmed; longer if cold.

4. Wash in water.

5. Aniline hydrochlorate, 2 per cent. aqueous solution, fifteen seconds.

6. Wash in water.

7. Absolute alcohol.

8. Xylol.

9. Xylol balsam.

To Stain Tubercle Bacilli in Celloidin Sections.—1. Stain rather lightly in alum-hematoxylin.

2. Wash in water.

3. Dehydrate in 95 per cent. alcohol.

4. Attach sections to slide by the ether-vapor method.

5. Carbol-fuchsin two to five minutes steaming.

6. Water.

7. Orth's discharging fluid (acid alcohol) one-half to one minute.

8. Wash thoroughly in several changes of water to remove acid completely and to bring back blue color to nuclei.

9. Alcohol 95 per cent. until fuchsin is entirely discharged.

10. Aniline followed by xylol; or blot and treat with xylol.

11. Xylol balsam.

The advantages of this method are—that the celloidin is colorless: the nuclei are stained blue; the rest of the tissue is colorless; the tubercle bacilli stand out in sharp contrast. It is sometimes an advantage to bring out the cell-protoplasm and the intercellular substance by staining the sections, after decolorization in alcohol, in an aqueous solution of orange G or methyl-orange for a few seconds.

The **bacillus of leprosy** stains more easily than the tubercle bacillus. Simple aqueous solutions of the aniline dyes are sufficient. The same methods can be employed as for tubercle bacilli. A method recommended by Flexner will be found very useful.

1. Stain in alum hematoxylin so as to get a sharp nuclear stain.

2. Wash in water.

3. Carbol-fuchsin two to five minutes steaming, or thirty to sixty minutes cold.

4. Water.

5. Treat on the slide with iodin solution one-half to one minute.

6. Water.

7. Blot; clear and differentiate in aniline oil.

8. Xylol; balsam.

Baumgarten gives the following differential stain for leprosy bacilli:

1. Stain six to seven minutes in a dilute solution of fuchsin (5 drops of a concentrated alcoholic solution to a watch-glass of water).

2. Discharge one-quarter minute in nitric-acid alcohol (nitric acid 1, alcohol 10).

3. Wash in water.

4. Contrast-stain in a saturated aqueous solution of methylene-blue.

5. Alcohol.

6. Xylol.

7. Balsam.

While leprosy bacilli stain readily by this method, tubercle bacilli will not stain in so short a time.

Syphilis Bacillus.—Lustgarten's Method.—1. Stain twenty-four hours at room-temperature and two hours in the thermostat at 40° in aniline-gentian-violet.

2. Wash off in absolute alcohol three to five minutes.

3. Decolorize (*a*) in a $\frac{1}{2}$ per cent. aqueous solution of permanganate of potassium, and then (*b*) a few seconds in an aqueous solution of pure sulphurous acid (strength not given).

4. Wash in water.

5. Alcohol.

6. Oil of cloves.

7. Canada balsam.

If the section is not entirely decolorized when the section is put into water, then the third step must be repeated until decolorization is complete. If desired, the sections can be stained in safranin after the fourth step.

Giacomi's Method.—1. Stain several minutes in hot aniline-fuchsin.

2. Wash out in very dilute aqueous solution of chlorid of iron.

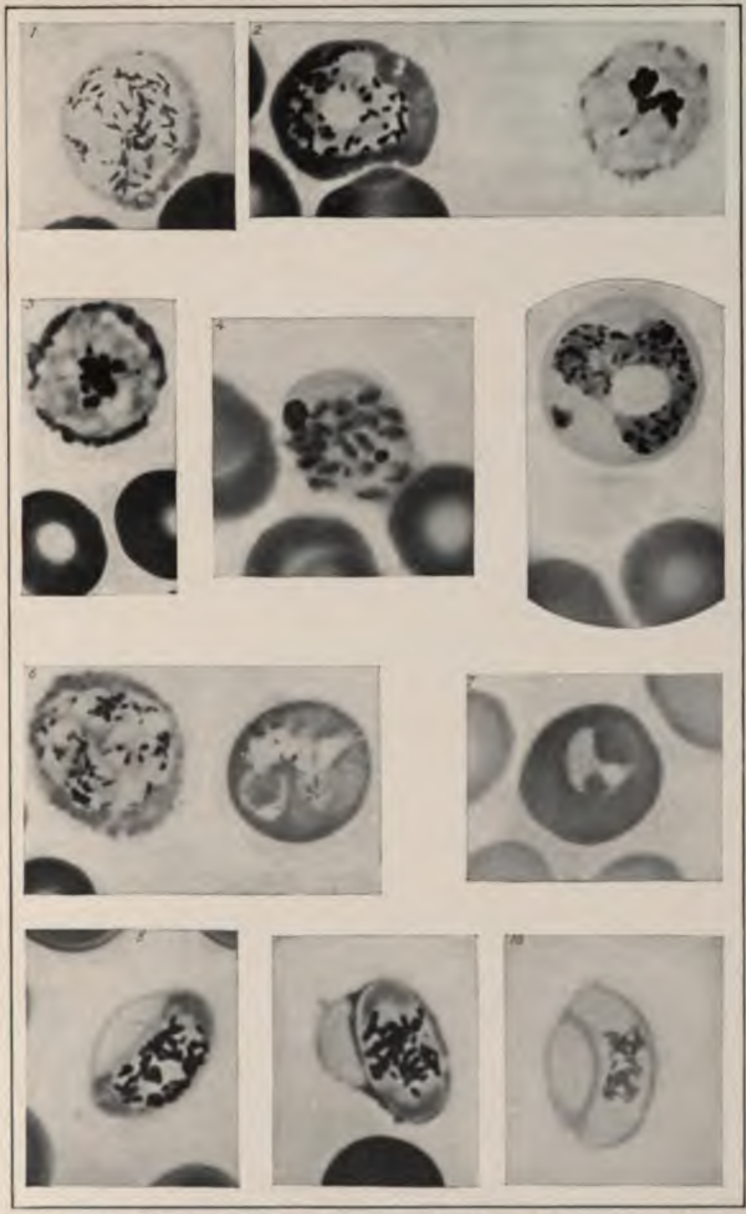
3. Decolorize in concentrated solution of chlorid of iron.
4. Wash out in absolute alcohol.
5. Xylol.
6. Xylol balsam.

The **smegma bacillus** is stained by Lustgarten's method, but not by the methods given for tubercle bacilli in tissues, because in all of them alcohol is used as well as an acid to effect the decolorization.

METHODS OF EXAMINATION OF ANIMAL PARASITES.

Protozoa.—Of the rhizopoda, the malarial organisms and the ameba coli are of much pathological interest.

Malarial Organisms.—Three varieties of the plasmodium malariæ have been described—namely, the tertian, quartan, and estivo-autumnal parasites. They develop within the red corpuscles and cause the destruction of the corpuscles affected. The earliest forms of the parasite appear in the blood during the latter part of the malarial paroxysm or shortly after it. At this time they appear as small, colorless, disc-shaped hyaline bodies which occupy but a small portion of the blood-corpuscles. They possess a varying degree of ameboid movement, the amount depending upon the type of the organism. These ameboid movements are best observed on the warm stage. During the process of development the parasites increase in size and more or less completely fill the red corpuscles containing them. Small particles of reddish-brown pigment are produced, during their growth, from the hemoglobin of the corpuscles in which the organisms are developing. These granules show varying degrees of motion, probably imparted to them by the movements of the parasites. At first the pigment appears to be scattered about in the corpuscle, but it is in reality in the extremities of the pseudopodia. Later it appears more evenly spread about in the periphery. Toward the end of the cycle of development the pigment collects in the center of the parasite; at this time the ameboid movements



Photographs of malarial parasites from fixed and stained preparations of blood: 1, 2, 3, 4, 5, various stages in the life-cycle of the tertian parasite; 6, in the left, an adult tertian parasite in a red blood-corpuscle; in the right, a young tertian parasite and a hyaline form of the æstivo-autumnal parasite in a red blood-corpuscle; 7, hyaline form of the æstivo-autumnal parasite in a red blood-corpuscle; 8, 9, 10, crescentic and ovoid forms of the æstivo-autumnal parasite in red blood-corpuscles. (Wright and Brown.)

have ceased, indications of segmentation occur, and the parasite nearly or completely fills the corpuscle. Oftentimes at this stage only a small portion of the corpuscle is visible at some point on the edge of the parasite.

The beginning of segmentation is indicated by a number of radial lines extending from the periphery of the parasite toward the central clump of pigment. Segmentation takes place, and the pigment is surrounded by a number of distinct segments which vary with the type of the organism. Each of these segments shows a central refractive spot which probably is the nucleus. At this time one notices small hyaline bodies, like those of the early stage in the development of the parasite, in some of the red blood-corpuscles. Oftentimes such a regular process of segmentation is not observed, but enough has been said to indicate the manner in which reproduction occurs. Segmentation is the indication of an approach of a paroxysm. Extra-cellular forms of the parasites are not infrequently seen. They may be fully-grown organisms which have destroyed the corpuscles that contained them, or they may be partly-grown organisms which have left the corpuscles. These free parasites are indistinct in outline and contain pigment. They possess ameboid movements, and may be considerably larger than a red blood-corpuscle. Various changes are observed in them:

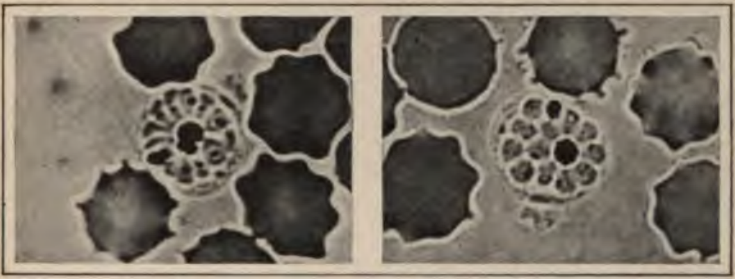


FIG. 112.—Two stages in the process of segmentation of a tertian malarial parasite in a red blood-corpuscle. Preparation of fresh blood, not stained (Wright and Brown).

1. They may increase in size until they become nearly as large as polymorphonuclear leucocytes. With the increase in size there is a gradual cessation in the movement of the pigment-granules until finally the organisms present the appearance of misshapen masses of protoplasm containing motionless pigment-granules.

2. They may undergo fragmentation and give off several small circular pigmented bodies.

3. Vacuolization may occur.

4. Flagellate forms may develop. One or more thread-like processes are thrust out from the organisms. These flagella may contain pigment, and may break away from the organism and move about among the corpuscles, looking not unlike the spirilla of relapsing fever.

The three varieties of parasites differ from each other in a number of ways. The chief differences are the length of the cycle of development; the size of the full-grown organisms; the difference in the refractibility of the organisms; the quantity, size, and color of the pigment-granules; the degree of ameboid movement; and the number and shape of the segments into which the full-grown organisms divide. In the earliest stage the varieties of organisms cannot be distinguished from each other.

The tertian parasite completes its cycle of development in about forty-eight hours. When it has attained its fullest growth it almost fills the corpuscle, which has become larger than normal. This organism is less refractive than either of the other two. The pigment-granules are more numerous, finer, and more reddish-brown in color; the ameboid movements are much more active; the segments are more irregular in shape and more numerous than those of the quartan parasite, varying from twelve to twenty in number.

The quartan appears to complete its cycle of development in from sixty-four to seventy-two hours. The full-grown organism does not fill completely the corpuscle, and the latter is not increased in size. The organism is more refractive than the tertian parasite. The pigment-granules are fewer in number, coarser, and have a darker-red color. The ameboid movements are slower; the segments are pear-shaped, more symmetrical, and less numerous than those of the tertian parasite, varying from six to twelve in number. Segmenting organisms are more numerous in the peripheral circulation than in the case of the tertian parasite.

The estivo-autumnal parasite cannot be studied so thoroughly in the peripheral circulation, because the later development and segmentation take place in the internal organs. The length of time required to complete its cycle of development is not so definitely settled. It appears to require from twelve to twenty-four hours, more or less. The full-grown organism is smaller than the tertian parasite, and the corpuscle which contains it is often smaller than normal and more or less distorted. The parasite is quite refractive. The pigment-granules are few in number and coarse. The ameboid movements are slow. After the duration of fever for from five days to a week or more, elongated, ovoid, or crescent-shaped bodies make their appearance. They are sometimes as large or larger than a red corpuscle. These bodies are not a result of segmentation, but appear to be a further development of the round hyaline bodies. They are highly refractive and contain granules of coarse pigment in the center. They lie usually at one side of the red corpuscles, the latter more or less completely filling the concavity between the two horns of the crescent. They may lie in the center of the corpuscles. Some of the apparently free ovoid bodies are turned in such a way as to present a convex surface toward the observer.

Double infections occur quite frequently in both tertian and quartan fever, and in the latter not infrequently triple infections occur. In the double infections two groups of parasites reach maturity on successive days and cause daily febrile paroxysms. In the triple infection of quartan fever three groups of organisms mature on successive days and cause corresponding paroxysms.

Methods of Examining the Blood for Malarial Organisms. —The organisms of malaria can be detected in fresh specimens of blood or in specimens of blood which have been fixed and stained. The examination of fresh specimens of blood is simpler and more reliable, because the development of the parasites can be observed.

The method employed in making cover-glass preparations of the blood has been thoroughly described (see preparation of cover-glass specimens in the Examination of the Blood, page 343).

In examining a fresh specimen of the blood for the malarial organisms a glass slide is substituted for one of the cover-glasses, and the cover-glass which has the drop of blood on its surface is dropped lightly upon the glass slide and allowed to remain there. The first four or five drops of blood should be quickly wiped away from the ear until a very small drop is obtained. Great care must be exercised to touch only the tip of the drop with the cover-glass, so as to avoid smearing the blood. If the blood is smeared on the cover-glass, the edges of the blood-drop will dry before the cover-glass can be transferred to the slide, and the blood will not spread. It is necessary that the blood should spread in a thin layer in order to study satisfactorily the individual corpuscles. If one desires to study the preparation for several hours, the edges of the cover-glass can be surrounded by melted paraffin or vaselin to exclude the air. The examination should be made with an oil-immersion lens. It should be remembered that the action of cold inhibits the ameboid movements of the parasites; it may be necessary, therefore, at times to warm the slide before examining the specimen. Evaporation not infrequently occurs, caused by the air penetrating beneath the cover-glass. This produces changes in many of the corpuscles which may be mistaken for hyaline bodies: the central depression becomes paler and less refractive than the periphery of the corpuscles; later a number of corpuscles contain small glistening points, and still later the corpuscles become crenated.

The method of procuring permanent cover-glass preparations is the same as that elsewhere described (see Cover-glass Preparations, page 343). Specimens may be fixed by heating or by a mixture of absolute alcohol and ether, equal parts. Numerous methods of staining have been employed. The following methods are simple and satisfactory: The fixed cover-glass preparation is stained from one to two minutes in a concentrated aqueous solution of methylene-blue. The staining solution should be filtered before using. The specimen is thoroughly washed in water, dried, and mounted in Canada balsam. This stains the organisms and the nuclei of the white corpuscles blue. The red corpuscles are unstained.

A good contrast-stain can be obtained with eosin and methylene-blue. The fixed specimens are stained for from thirty seconds to five minutes in a ½ per cent. solution of eosin in 60 per cent. alcohol, washed in water, dried, and placed for from thirty seconds to one minute in a concentrated aqueous solution of methylene-blue, washed, dried, and mounted in Canada balsam. The red corpuscles are stained a bright red by the eosin, and the organisms and the nuclei of the white corpuscles are stained blue.

Excellent results are obtained by Romanowsky's method. This method is as follows: A saturated aqueous solution of methylene-blue and a 1 per cent. aqueous solution of eosin are kept separately. The older the methylene-blue solution the better the results. The specimen is then heated not less than thirty minutes at a temperature of from 105° to 110° C. The staining mixture is made just before it is to be used. To 1 part of the filtered methylene-blue solution are added about 2 parts of the eosin solution. This mixture is carefully stirred with a glass rod, but not filtered, and poured into a watch-glass. The cover-glass preparations are allowed to float upon the top of the fluid with the blood surface down. The specimens are covered by another inverted glass, and the whole by an inverted cylinder which is moistened upon the inside. Good specimens are obtained in from one-half to three hours. Romanowsky believes that he obtains thus three colors: the red corpuscles stained red by the eosin, the malarial parasites a Prussian blue by the methylene-blue, and the nuclear chromatin a violet color (a neutral stain). Thayer and Hewitson claim to have obtained their best results by means of a slight modification of Romanowsky's method. The modification consists in fixing the specimens in absolute alcohol for from ten to twenty minutes instead of employing heat.

In the fixed and stained specimens there is always a possibility of mistaking small particles of methylene-blue, which have passed through the filter-paper, for malarial organisms. Sometimes these particles lie on the red corpuscles, and it is difficult to determine whether they are inside of the corpuscles or lying on the surface.

NOTE.—The description of the development of the parasites is abstracted from Thayer and Hewitson's *The Malarial Fevers of Baltimore.*

Futcher's Method for Staining Malarial Parasites.—Make cover-glass preparations from the blood (as described on page 343); let dry in the air and fix in a 1/4 per cent. solution of formaldehyde in 95 per cent. alcohol. This solution must be freshly prepared. A convenient way to make it is to add four or five drops of a 10 per cent. aqueous solution of formaldehyde to 10 c.c. of 95 per cent. alcohol just before use. For staining, a solution of thionin is used. This solution is made as follows:

Sat. solution thionin in 50 per cent. alcohol,	20 c.c.;
2 per cent. aqueous solution of carbolic acid,	100 c.c.

This mixture improves with age. The details of the method are as follows:

1. Fix in the formalin solution one minute.
2. Wash in water.
3. Dry thoroughly with filter-paper.
4. Stain in thionin solution ten to fifteen seconds.
5. Wash in water, dry, and mount in balsam.

The parasites are stained reddish-violet by this method. It is especially good for the ring-shaped bodies of the æstivo-autumnal infection.

Ameba Coli.—In cases of dysentery suspected of being due to the ameba coli (Fig. 113) the stools are best examined as soon as voided, although the amebæ will sometimes remain active in stools even over twenty-four hours old. A warm stage during the examination is an advantage, but not a necessity. A drop of the fluid material, preferably that containing mucus or blood, is placed on a slide and lightly covered with a cover-glass. If the slide is cold and the organisms do not move, warm the slide gently and the movements of the amebæ will often start up. Pus from abscesses due to the amebæ is examined in the same way. A positive diagnosis rests on the presence of the characteristic large, pale cells, consisting of nucleus, granular endosarc, and hyaline ectosarc, and on the movements of the protoplasm, which projects itself more or less actively in the form of pseudopodia.

In hardened preparations the nuclei of the amebæ do not stain with the ordinary nuclear stains, such as alum-hematoxylin and methylene-blue. The following method of staining them has been found to give very satisfactory results and to render the recognition of the organisms easy:

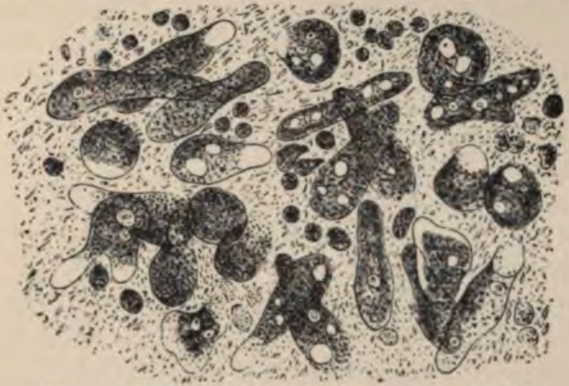


FIG. 113.—Amebæ coli in intestinal mucus (after Lösch).

Differential Stain for the Ameba Coli (*Mallory*).—1. Harden in alcohol.

2. Stain sections in a saturated aqueous solution of thionin three to five minutes.

3. Differentiate in a 2 per cent. aqueous solution of oxalic acid for one-half to one minute.

4. Wash in water.

5. Dehydrate in absolute alcohol.

6. Clear in xylol.
7. Xylol balsam.

The nuclei of the amebæ and the granules of the mastzellen are stained brownish red; the nuclei of the mastzellen and of all other cells are stained blue.

Excellent results were obtained by this method with bits of the purulent discharge from a so-called amebic abscess of the liver. After hardening in 95 per cent. alcohol, small fragments the size of a pin-head and less were stained as above directed, and teased apart after they were in the balsam. The reddish nuclei stood out so sharply in the bluish background of fragmented nuclei and granular detritus that they were easily picked out with the high dry power.

The results obtained with feces examined in the same way or after imbedding in celloidin were much less satisfactory, for the reason that various substances in the feces precipitate the thionin in the form of reddish crystals and give rise to deceptive pictures. A similar differential stain can be obtained by Unna's method for staining the granules of mastzellen (see page 303).

Other protozoa, such as the cercomonas and trichomonas, are best examined in fresh preparations.

Sporozoa.—The coccidium oviforme should be examined both fresh in cover-slip preparations and in sections after hardening. The cyst-forms often stain well by the tubercle-bacillus method. For studying all the stages in the development of the organism Pianese recommends highly his special fixing reagent and stains (see p. 249). Other sporozoa should be studied in the same manner. Cover-slip preparations are often useful.

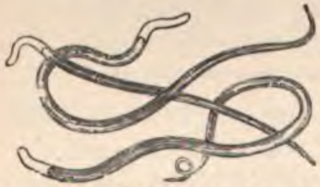


FIG. 114.—Filaria sanguinis hominis (after von Jaksch).

Round-worms.—The embryos of the *filaria sanguinis hominis* (Fig. 114) are examined for in suspected cases by mounting a drop of the fresh blood or of the chylous or bloody urine on the slide and examining under a low power. They are readily detected when present on account of their very active movements. Six species have been described, but the filaria nocturna is the only one that is known to be pathogenic. The blood should be examined during the resting hours of the patient, as at night for day-workers and during the day for night-workers. Permanent specimens can be made by fixing ordinary cover-slip preparations of the blood or chylous fluid by heat or by the use of a saturated solution of corrosive sublimate, and staining for a few seconds with Löffler's methylene-blue or with a 2 per cent. aqueous solution of thionin.

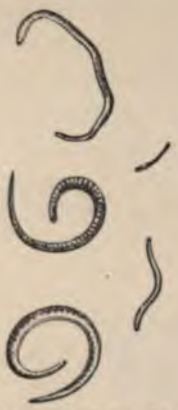


FIG. 115.—Living embryos (Heller).

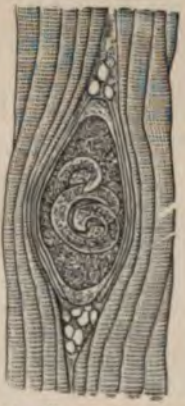


FIG. 116.—Encapsulated trichina (Leuckart).

Trichinæ (Figs. 115, 116) are obtained from the fresh muscle by means of teasing. A quick method is to squeeze small bits of tissue between two slides and examine with a low power. Pieces of muscle nearest the insertion of the tendon are chosen from the diaphragm or from the muscles of the jaws. Encapsulated and calcified trichinæ are cleared up by means of acids.

In hardened tissues the trichinæ are best studied in longitudinal sections of the muscle-fibers.

The other round-worms which sometimes occur in the intestinal tract can be recognized with the naked eye. Their eggs must be looked for with the microscope.

Tape-worms.—It is not always easy to recognize the kind of tape-worm by a single segment passed with the feces, because the uterus, which furnishes the most characteristic points of difference, is not developed in the young segments and is atrophied in the old ones. When the whole worm is obtained the problem is much simpler. The uterus is best made out by squeezing a segment between two slides and

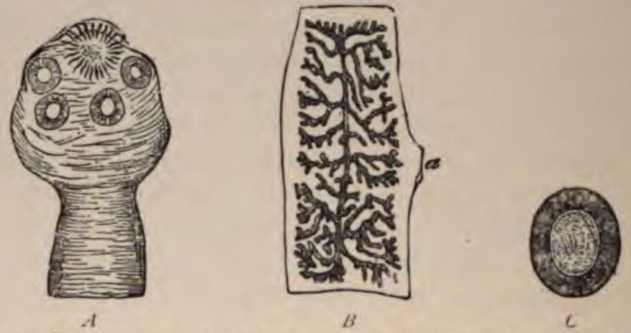


FIG. 117.—Tænia solium: *A*, head enlarged; *B*, ripe joint, × 6; *C*, egg of tænia solium (Heller).

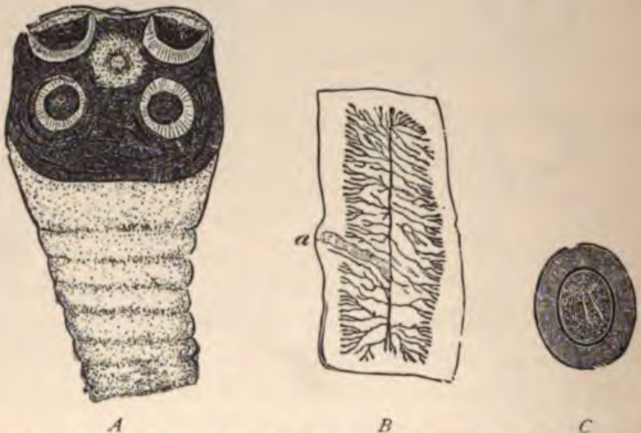


FIG. 118.—Tænia mediocanellata: *A*, head darkly pigmented; *B*, ripe joint, × 6; *C*, egg of tænia mediocanellata.

holding it up to the light. The heads are examined under the microscope in water, salt solution, or glycerin.

Tænia Solium (Fig. 117).—Head has four suckers and a circle of hooklets; uterus is noticeably but little branched. The genital tract opens laterally. The eggs develop into the cysticerci cellulosæ, which are not infrequently found in man. The scolex is obtained for examination by tearing open the cyst and examining the inner wall. The suckers and hooklets are best studied after mounting fresh and pressing under a cover-glass.

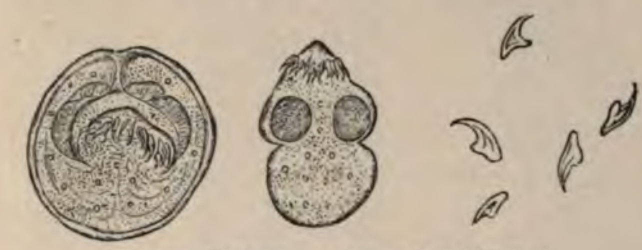


FIG. 119.—Echinococcus: solices, hooks (Heller).

Tænia Mediocanellata s. Saginata (Fig. 118).—Head has four strong suckers, but no hooklets; uterus is very much branched, segments show marked muscular development. The genital tract opens laterally. The eggs develop into *cysticerci*, which do not occur in man.

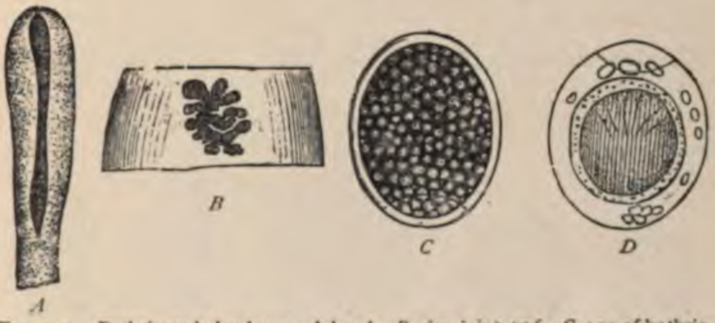


FIG. 120.—Bothriocephalus latus: *A*, head; *B*, ripe joint, × 6; *C*, egg of bothriocephalus latus (Heller); *D*, egg with developed embryo (Leuckart).

Tænia Echinococcus (Fig. 119) occurs in dogs. The echinococcus cysts which occur in man are recognized by the very characteristic laminated structure of the cyst-wall.

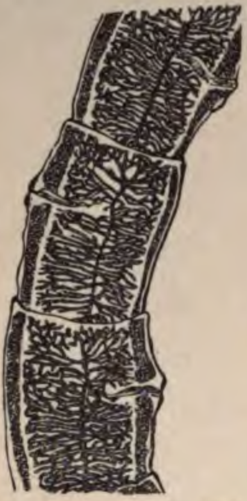


FIG. 121.—Segments o. tænia saginata (after Stein).

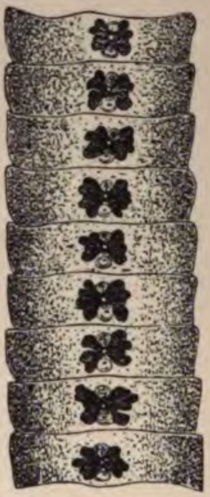


FIG. 122.—Segments of bothriocephalus latus (after Stein).

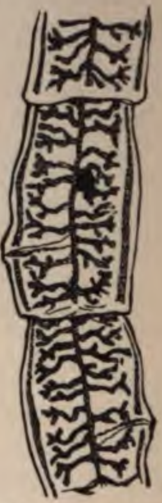


FIG. 123.—Segments of tænia solium (after Stein).

The heads of the scolices have four suckers and a double circle of hooklets.

Bothriocephalus Latus (Fig. 120).—The opening of the genital tract lies in the median line. The head is flattened, and has two small suckers situated at the sides.

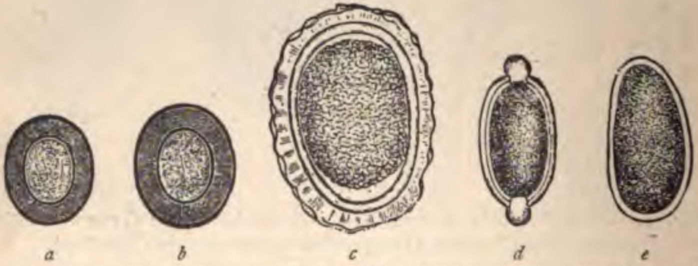


FIG. 124.—Comparative size of eggs of intestinal parasites: *a*, tænia solium; *b*, tænia mediocanellata; *c*, ascaris lumbricoides; *d*, trichocephalus dispar; *e*, oxyuris vermicularis (after Strümpell).

CLINICAL PATHOLOGY.

THE material received at the pathological laboratory from clinical cases consists largely of new growths and lesions produced by infectious, chemical, or traumatic agents—to a less extent of fluids from various natural or artificial cavities or secretions and eliminations from the respiratory, genito-urinary, and gastro-intestinal tracts. In the following pages a brief synopsis is given of what may be expected in the various fluids and eliminations, and the more important chemical tests required for the recognition of certain substances are added.

Gross specimens removed at operations should be treated as parts of autopsies, and dissected or otherwise examined bacteriologically and histologically in an equally careful manner. For the finer study of cancers and other new growths the recently published methods of Pianese should be employed (see page 275).

Examination of Tissues from Clinical Cases for Diagnosis.—Tumors of any size or large pieces of tissue present no difficulties. There is plenty of material to examine fresh or after fixing in a variety of ways. The small bits removed with a Mixter punch or cut from the edge of a growth, of which a diagnosis is wanted immediately, sometimes prove troublesome. Frozen sections of the fresh tissue are often sufficient. Sometimes it is better to harden for an hour or more in formaldehyde and then to make frozen sections.* Often it is wisest to harden in strong alcohol and then to make razor sections or to imbed in celloidin. The whole process of hardening, imbedding, and sectioning can easily be carried through in twenty-four hours with small pieces of tissue. Imbedding in paraffin is sometimes preferred. In cutting sections of small pieces it is important to mount them if possible, so that the cut sections will show proper relations—*i. e.* vertical sections through the skin, uterine mucous membrane, etc.—otherwise confusing pictures will often be presented. It is important to know, in

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[1] For a rapid method for frozen sections, see p. 417.

regard to pieces of tissue sent for diagnosis, from what part of the body they come.

A hematoxylin-and-eosin stain will be found the most generally useful for hardened sections.

Uterine Scrapings.—Small pieces may be examined fresh in frozen sections or after hardening for one or more hours in formaldehyde. Better results are obtained by hardening in alcohol and imbedding in celloidin or paraffin. Where the fragments are small, it is advisable to mass them together on a small piece of filter-paper and to harden in strong alcohol. The mass can then be imbedded in celloidin and cut as one piece of tissue. A hematoxylin-and-eosin stain is the best, because the eosin brings out the smooth muscle-fibers prominently, so that any invasion of the muscular coat by a malignant growth is more readily made out—a valuable help in the diagnosis of malignant adenoma.

Examination of Fluids obtained by Puncture.—The **transudations** obtained largely from the serous cavities are non-inflammatory in origin. They are usually of a transparent, pale-yellow color with slightly greenish tint, alkaline in reaction, and deposit on standing a slight flocculent coagulum.

The *specific gravity*, to be taken at room-temperature, varies according to the origin of the fluid. According to Reuss, it is below 1015 in hydrothorax; below 1012 in ascites; below 1010 in anasarca; below 1008.5 in hydrothorax.

The amount of *albumin* in hydrothorax is always under 2.5 per cent., and in ascites between 1.5 and 2 per cent. Microscopically, a few leucocytes, usually fatty degenerated and rarely desquamated endothelial cells, are found.

The **exudations** are of inflammatory origin, and are also generally obtained from the serous cavities. From their various microscopic appearances they are divided into serous (fibrino-serous), hemorrhagic, purulent, and gangrenous. The specific gravity of all is over 1018; the reaction is always alkaline. On standing they deposit a varying amount of sediment. Examinations for organisms should always be made (see page 89). Occasionally a peculiar opalescent layer, due to cholesterin crystals, forms on the surface of fluids which come from old cases of pleurisy.

Serous Exudations.—The fluid, which immediately after removal is slightly cloudy and yellowish in color, deposits more or less quickly a flocculent or dense coagulum. Microscopically, the coagulum shows a dense meshwork of fibrin and numerous polynuclear leucocytes.

Hemorrhagic Exudations.—The sero-fibrinous exudation is colored a lighter or darker red according to the amount of blood present. Microscopically, the same elements are found as in the serous exudations, plus a marked increase of red blood-globules, which are usually well preserved, but in old exudations may be more or less decolorized.

Aside from injuries, hemorrhagic exudations are most common in connection with tuberculosis and new growths, so that their microscopic examination is of much diagnostic and prognostic value.

For the examination of tubercle bacilli see page 175. In this form of exudation it is rarely possible to demonstrate them. On the other hand, it is not infrequently possible to make the diagnosis of a malignant growth, especially of cancer, from the examination of the sediment. No cell is significant of cancer or other neoplasm, but the occurrence of numerous cells which vary greatly in form is suspicious. The cells from new growths are often unusually large, up to 120μ, frequently contain one or more vacuoles, and usually lie in clumps. Large drops of fat are also considered suspicious.

A positive diagnosis can only be made by obtaining bits of tissue which show the structure of the new growth, such as the atypical alveolar arrangement of the cells in cancer.

Purulent exudations appear more or less thick and yellow, and deposit a corresponding layer of pus. Microscopically, they present no peculiarities other than the organisms to which they are due. Among the etiological factors actinomyces must always be thought of in puzzling cases.

Putrid exudations occur in the pleural and peritoneal cavities in consequence of gangrenous masses breaking into them and from stomach or intestinal ulcerations, from new growths, occasionally from no clear cause. The fluid resulting from the perforation of a gastric ulcer may show yeast-cells and sarcinæ, and give an acid reaction.

Lumbar Puncture.—The diagnostic value of lumbar puncture has been sufficiently demonstrated. Not only is it possible to diagnosticate inflammation of the meninges, but the character and cause of the inflammation may usually be demonstrated if the examination of the fluid is properly performed. In a number of cases of general infection in which there was no inflammation of the meninges a diagnosis has been made by means of cultures taken from the cerebro-spinal fluid. Finally, a number of cases of hemorrhage into the brain and spinal canal have been diagnosticated by lumbar puncture.

The operation and the subsequent examination of the fluid should be as carefully performed as any other bacteriological investigation in order to obtain accurate results. The back of the patient and the operator's hands should be made sterile. The needle should be boiled for ten minutes. The patient should lie on the right side, with the knees drawn up, and with the uppermost shoulder so depressed as to present the spinal column to the operator. This position permits the operator to thrust the needle directly forward rather than from the side. An antitoxin needle 4 cm. in length, with a diameter of 1 mm., is well adapted for infants and young children. A longer needle is necessary for adults and children over ten years of age.

Aspiration of the fluid is not necessary, but some operators prefer to attach a hypodermic syringe to the needle to afford a better grasp for the hand. In this case the syringe would have to be detached to allow the fluid to flow. The additional manipulation, and possibly the defective sterilization of the syringe, might impair the subsequent bacteriological examination.

The puncture is generally made between the third and the fourth lumbar vertebræ; sometimes between the second and third. The thumb of the left hand is pressed between the spinous processes, and the point of the needle is entered about 1 cm. to the right of the median line and on a level with the thumb-nail, and directed slightly upward and inward toward the median line. Care must be exercised to prevent the point of the needle from passing to the left of the median line and striking on the bone. At a depth of 3 or 4 cm. in children and 7 or 8 cm. in adults the needle enters the subarachnoid space, and the fluid flows usually by drops. If the point of the needle meets with a bony obstruction, it is advisable to withdraw the needle somewhat, and to thrust again, directing the point of the needle toward the median line, rather than to make lateral movements, with the danger of breaking the needle or causing a hemorrhage. The smallest quantity of blood obscures the macroscopic appearance of the fluid by rendering it cloudy. The fluid is allowed to drop into an absolutely clean test-tube which previously has been sterilized by dry heat to 150° C. and stoppered with cotton. The fluid should be allowed to drop into the tube without running down the sides. From 5 to 15 c.c. of fluid is a sufficient quantity for examination.

Cultures on blood-serum should be made at once from the fluid. The test-tube is then held toward the light and gently shaken; by this means the slightest cloudiness can be detected.

In meningitis there is always an exudation of cells which makes the fluid more or less cloudy. The degree of cloudiness is to some extent proportionate to the amount and character of the exudation. In tubercular meningitis the amount of cellular exudation is sometimes so slight that the fluid appears clear unless examined carefully. A guinea-pig should be inoculated with some of the fluid as a control-experiment. The inoculation should be made a short time after withdrawing the fluid before the formation of fibrin occurs, because the web-like coagulum of fibrin holds the tubercle bacilli in its meshes. After standing for several hours more or less fibrin forms in the fluid, and contains the cells in its meshes. The supernatant fluid is left clear. Cover-glass preparations should be made from this sediment, dried by passing through the flame of an alcohol lamp or Bunsen burner, and stained with methylene-blue, which stains the nuclei of the cells and any pathogenic bacteria which may be present.

In tubercular meningitis the predominant cell is the lymphoid cell. In purulent meningitis the polynuclear leucocyte predominates. Cover-glass preparations may also be stained for tubercle bacilli by appropriate methods of staining.

It is often necessary to examine twenty or more preparations, however, before finding the bacilli. The albumin should be quantitated. Normally, the cerebro-spinal fluid contains from $\frac{1}{40}$ to $\frac{1}{100}$ of 1 per cent. or less. In meningitis the amount is increased from $\frac{1}{30}$ to $\frac{1}{10}$ of 1 per cent., or often more. Percentages of albumin down to $\frac{1}{100}$ of 1 per cent. can be estimated by the ferrocyanide-of-potassium-and acetic-acid test and a centrifugal machine. Take $3\frac{1}{2}$ c.c. of a 20 per cent. solution of ferrocyanide of potassium, $1\frac{1}{2}$ c.c. of acetic acid, and 10 c.c. of the fluid in which the albumin is to be quantitated. The mixture of reagents and fluid is poured into a conically-shaped graduated glass vessel designed for the purpose and centrifugalized. Sugar is rarely present and has no diagnostic value.

Ovarian and Parovarian Cysts.—The simple cysts of the ovary due to distention of Graafian follicles or to cystic change of corpora lutea, and the parovarian cysts contain a thin, clear, serous fluid of low specific gravity.

The contents of the multilocular and papillary adeno-cystomata of the ovary are usually tenacious and mucous, of very varying specific gravity, from 1005–1050, but usually between 1020 and 1024. The fluid generally contains much albumin and is rich in metalbumin, which is precipitated by alcohol, but not by acetic acid, nitric acid, or boiling, so that it can readily be distinguished from mucin. Before making the test the albumin must be removed.

The cyst-contents are usually yellowish, but sometimes may be dark-red or chocolate-colored. Microscopically, red and white blood-globules, occasionally blood-pigment and cholesterin crystals, often fat-granules and large vacuolated cells, are found in the cyst fluid. Bizzozero considers cylindrical epithelial cells, ciliated and beaker cells, and colloid concretions especially important from a diagnostic point of view.

Pancreatic Cyst or Fistula.—The fluid obtained from a permanent fistula or large cyst of the pancreas contains much less solids than the normal pancreatic juice, and the trypsin ferment may be present in very small amount or possibly be entirely wanting. The fluid is colorless, alkaline in reaction, and has a specific gravity of about 1011. It is characterized by three distinct properties on which its recognition depends—namely:

1. It splits up fat into fatty acids and glycerin. Mix together equal parts of neutral olive oil and the alkaline fluid. Test with litmus-paper. Place the mixture in the incubator at 37° C., and test from time to time. If the fluid is pancreatic, an acid reaction will be obtained in twelve to eighteen minutes.

2. It transforms starch into sugar. Place in the incubator equal parts of a 1 per cent. aqueous solution of starch and of the fluid to be tested. In ten to twenty minutes test for sugar with Fehling's solution.

3. It digests fibrin in an alkaline solution (trypsin ferment). Place some fibrin in the alkaline fluid and set it in the incubator. In one-half to one hour examine for peptones by the *biuret test.* Add caustic potash or soda and a few drops of a dilute solution of sulphate of copper. If peptones are present, a beautiful reddish-violet color will be produced.

Dropsy of the Gall-bladder.—Puncture is generally not advisable. The fluid is usually colorless and mucoid or serous in character. All trace of biliary constituents may have disappeared. According to Lenhartz, numerous colon bacilli are usually present.

Hydronephrosis and Renal Cysts.—The fluid is almost always clear as water, rarely reddish or yellow. Specific gravity always under 1020 (usually between 1010 and 1015). Urea and uric acid are generally present, but may be absent. (Small amounts of urea are sometimes present in ovarian cysts.) Albumin is slight in amount. Microscopically, almost nothing is found.

Echinococcus Cysts.—The fluid is perfectly clear, free from albumin, and contains a little succinic acid and much chlorid of sodium. The specific gravity varies between 1008 and 1013.

Microscopically, often no traces of morphological elements can be found. Occasionally, however, hemosiderin granules or chlolesterin crystals occur, or the characteristic structures from which a positive diagnosis can be made—namely, scolices, hooklets, or pieces of cyst-membrane.

A positive diagnosis from a chemical examination depends on showing—

1. The absence of albumin.

2. The presence of chlorid of sodium.

Evaporate a drop of the fluid slowly on a slide, so as to get the characteristic crystals of chlorid of sodium.

3. The presence of succinic acid.

Acidify a little of the fluid with hydrochloric acid and evaporate to dryness. Extract the residue with ether. The crystallized material left on the evaporation of the ether, if dissolved in water, will give a rust-colored, gelatinous precipitate with sesquichlorid of iron if succinic acid be present.

Examination of the Sputum.—The secretion raised from the air-passages by coughing is almost invariably contaminated with the secretion of the naso-pharynx and with particles of food from the mouth. In examinations of sputum these contaminations must always be borne in mind. The amount raised varies from a few c.c. to one or even several liters in twenty-four hours.

The macroscopic appearances of the sputum depend on the varying proportions of mucus, pus, blood, and serum present. The tenacity is mainly due to the mucus. The reaction is usually alkaline.

The general color, consistency, and separation into layers is best seen after the sputum has stood for some time in a tall glass. For more careful macroscopic examination small portions of the sputum are transferred to flat glass dishes, where they are spread out thinly by needles and examined over black or white paper. Porcelain plates painted black or black paper itself can be used. The latter method is convenient, because the sputum can be burned up with the paper.

The constituents of the sputum which may be recognized macroscopically are few in number, and not so important as those which may be found microscopically.

Macroscopic Examination.—1. *Caseous Masses.*—In the sputum from tubercular cases small, opaque, yellowish-white masses from the size of a pin-head to that of a small pea can occasionally be found, which spread out beneath a cover-glass like a bit of cheese. They are small caseous masses which are valuable for microscopic examination because they usually contain tubercle bacilli and elastic fibers.

2. *Fibrinous casts of the bronchioles* can usually be found in the sputum from the third to the seventh day in cases of acute lobar pneumonia. They appear as yellowish-white or reddish-yellow threads, 2 to 3 mm. thick and ½ to several cm. long, and are often branched. The large ones are often rolled into balls, and show best after being shaken in water. Casts of the bronchi are found in cases of fibrinous bronchitis.

3. *Curschmann's spirals* (Fig. 125) of twisted threads of mucus enclosing epithelial cells and leucocytes occur rarely except in bronchial asthma. They appear macroscopically as grayish-white or whitish-yellow masses or threads, ½ to 1½

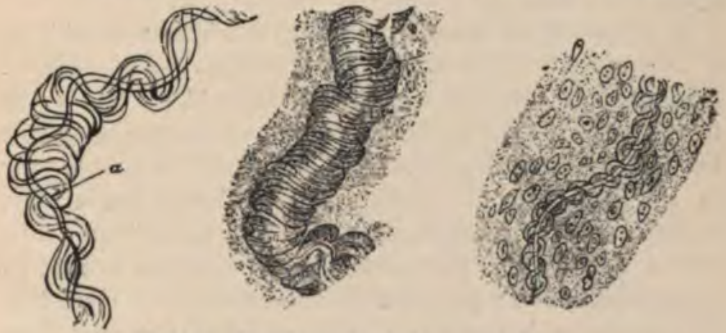

FIG. 125.—Curschmann's spirals: *a*, central fiber.

mm. thick and ½ to 8 cm. long, and often show a visible spiral arrangement.

4. *Dittrich's Plugs.*—These are whitish-yellow masses from the size of a pinhead to that of a bean, which are formed in cases of putrid bronchitis and of gangrene of the lung. They have a very fetid odor, a cheesy consistency, and are rather easily compressed. Besides organisms they contain numerous fat-crystals.

5. *Shreds of tissue* are found almost solely in gangrene of the lung, and are best recognized with the microscope.

6. *Concretions*, portions of cysticercus membrane, etc. are rare in the secretion from the lungs.

Microscopic Examination.—Microscopically, the sputum may show various kinds of cells, fragments of tissue, including elastic fibers, vegetable and animal parasites, and crystals.

They will be taken up in order:

1. *Red Blood-globules.*—In fresh hemorrhages they appear normal, often in rouleaux. In old sputa many have lost their color.

2. *White blood-globules* are almost invariably polynuclear, and the majority of them contain neutrophilic granules. In asthma, however, numerous eosinophilic and rather numerous basophilic leucocytes are regularly found. The leucocytes often contain pigment- or fat-granules.

3. *Epithelial Cells.*—Pavement, cylindrical, and ciliated cells are found. The first come from the naso-pharynx; the others usually from the trachea and bronchi, but may come from the nose. Desquamated alveolar epithelium is difficult to demonstrate. The pigmented cells found almost wholly in chronic passive congestion of the lungs are chiefly, perhaps entirely, desquamated alveolar epithelium. The pigment appears as yellowish, yellowish-red, or brownish-red granules or as yellow diffuse pigmentation. Occasionally, however, it surrounds granules of carbon, and then appears brownish or grayish black. The pigment is derived from the blood, and will usually give the iron reaction (see page 376), but very young or old pigment will not.

4. *Fatty Detritus.*—Fat-drops are frequently found, due to the fatty degeneration of cells.

5. *Elastic fibers* (Fig. 126) occur singly, but more often as a network. They are recognized by their sharp, dark outlines, due to their high degree of refractiveness, and by their marked degree of resistance to acids and alkalies by which other like tissues, such as connective-tissue fibers, are destroyed. Elastic fibers are most abundant in the caseous masses above mentioned. When these masses cannot be found, the thicker portions of the sputum are squeezed between a slide and cover-glass or between two slides, and examined with a low power. The examination is rendered easier by mixing a little sputum with a 10 per cent. solution of caustic potash or soda. In certain cases it is necessary to mix together equal parts of the sputum and 10 per cent. caustic potash or soda, and to boil the mixture until the sputum is dissolved. The solution is then mixed with four times its own volume of water and allowed to stand for twenty-four hours, when the sediment can be examined for the elastic fibers.

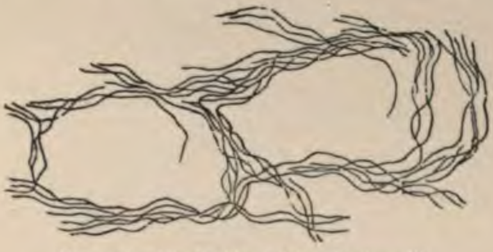

FIG. 126.—Elastic fibers (after Strümpell).

Vegetable and Animal Parasites.—Of the vegetable parasites, the most important is the tubercle bacillus (for its examination see page 175). Other bacteria sometimes examined for are the pneumococcus, the influenza bacillus, and actinomyces.

Of the animal parasites, the ameba coli is sometimes found secondary to an hepatic abscess which has perforated into the lung (see page 396). Portions of the membrane of an echinococcus cyst or the hooklets from the head may be found in the sputum, but infection with this parasite is very rare in this country.

Of the crystals which occur in sputa, the most important are the Charcot-Leyden crystals, found mainly in bronchial asthma, and the crystals of the fatty acids, of cholesterin, and of hematoidin. Tyrosin and leucin are much more rare.

The **Charcot-Leyden crystals** are colorless, elongated octahedra of varying size, soluble with difficulty in cold water, insoluble in alcohol, ether, chloroform, and dilute saline solution.

Hematoidin crystals occur as ruby-red rhombic plates or columns.

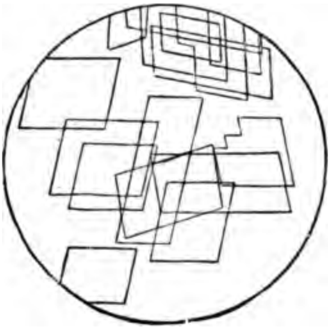

FIG. 127.—Crystals of cholesterin (after Strümpell).

Cholesterin crystals (Fig. 127) occur as the well-known small and large rhombic plates.

The **fatty-acid crystals** occur as long, pointed needles, either singly or in groups. They are easily soluble in ether or hot alcohol, insoluble in water and acids.

Examination of the Gastric Contents.—The microscopic examination of the contents of the stomach is much less important than the chemical. Fresh blood is easily recognized by the microscope. Disintegrated blood must be examined for chemically by the hemin test, as follows:

Mix a little of the suspected material with a crystal or two of common salt, or place it on the thin layer of salt formed by slowly evaporating a small drop of normal salt solution on a slide. Cover with a cover-glass, and run in enough glacial acetic acid to fill up the space between slide and cover. Warm the slide over a flame for three-quarters to one minute until bubbles arise, adding more glacial acetic acid as evaporation takes place, until a faint reddish-brown tint appears. Then let the acetic acid evaporate entirely, and run glycerin in from the edge of the cover-glass. Microscopic examination will show dark-brown rhombic plates or columns of hemin if blood is present.

Shreds of tissue or bits of mucous membrane are sometimes found in the vomitus or removed by means of a stomach-tube. Examination of them in the fresh condition, or, more satisfactorily, in stained sections after hardening and imbedding, will sometimes give definite information in regard to the condition of the mucous membrane, or render possible the diagnosis of a malignant growth.

Examination for Free Hydrochloric Acid.—Of the following tests, that with Congo-paper is the quickest and easiest, but shows only that a free acid is present. To prove that the free acid is hydrochloric acid the phloroglucin-vanillin test or one of the others is necessary.

1. *Congo-paper* is turned blue by free acids only. Free hydrochloric acid turns it of a cornflower-blue, a tint obtained with lactic acid only when in much greater concentration than is ever present in the stomach. Congo-paper is used simply by dipping it into the stomach-contents, preferably after filtration.

2. *Günzburg's Test with Phloroglucin-vanillin.*—The solution consists of—

Phloroglucin,	2;
Vanillin,	1;
Absolute alcohol,	30.

Three or four drops of this solution are placed with an equal amount of the filtrate from the stomach-contents in a porcelain dish and carefully heated over a small flame. Keep the dish in constant motion, and do not allow the mixture to boil, because boiling prevents the reaction from taking place.

If free hydrochloric acid is present, a rose-red mirror is produced. The phloroglucin-vanillin solution does not always keep well, so that it is best to keep alcoholic solutions of phloroglucin and of vanillin in separate bottles, and to mix together one or two drops of each when required.

3. *Boas' Resorcin Test.*—The solution consists of—

Resublimed resorcin,	5;
Cane-sugar,	3;
Alcohol, 94 per cent.,	ad 100.

It is used in the same manner as the phloroglucin-vanillin test. A similar but more transient mirror is produced.

Töpfer's Dimethyl-amido-azo-benzol test is highly recommended by Simon as superior to the phloroglucin-vanillin test. "One or two drops of a 0.5 per cent. alcoholic solution is added to a trace of the gastric contents, which need not be filtered; in the presence of free HCl a beautiful cherry-red color develops, which varies in intensity according to the amount of free HCl present."

Examination of the Feces.—In examining for tapeworms and their eggs it is often best to dilute the feces with water, and then to examine the sediment both macroscopically and under the microscope. (For ameba coli see p. 396.) The other protozoa are best looked for in fresh slide preparations.

For the cholera vibrio see p. 180; for the typhoid bacillus see p. 165.

The membranous casts sometimes found in feces consist almost wholly of mucus, cylindrical epithelial cells, and leucocytes. Bits may be examined fresh, or the casts may be hardened and sections made and stained after imbedding in celloidin.

Examination of the Urine.—Only those points are mentioned which come within the province of the pathologist.

Of the animal parasites, the echinococcus and the filaria sanguinis hominis are the only important ones, and they are very rare (see pp. 398, 402).

Of the vegetable parasites, tubercle bacilli and gonococci are the most common; actinomycetes are very rare.

New growths in the kidneys are accompanied with hemorrhage in less than half of the cases, while new growths in the bladder almost invariably give rise to it. Fragments from new growths in the bladder are rare. A diagnosis of malignant disease from cells only is impossible. Pieces of tissue which show on microscopic examination the characteristic structure of cancer or other neoplasm must be obtained in order to render a diagnosis possible from the pathological (but not from the clinical) standpoint.

A Rapid Method of Making Permanent Mounts of Frozen Sections.—This is a modification, by J. H. Wright, of the method of T. S. Cullen.

1. Place the specimen in 10 per cent. solution of formalin for two hours or more. If there is not time enough for this, the specimen may be boiled in the same solution, in a test-tube over the Bunsen flame, for two or three minutes. This does not give as good histological detail as does the procedure first mentioned. The specimen should not be more than 5 mm. in thickness.

2. Rinse in water.

3. Cut frozen section (see page 229). The sections must be as thin as possible without being so thin as to fall apart. Sufficiently thin sections may be obtained if the edge of the knife is in good condition. It should be given a smooth edge by rubbing on a razor-strop.

4. Float the section on the slide and arrange it smoothly, removing superfluous water.

5. Place on the section a sheet of smooth cigarette-paper, and then press on this, over the section, with a pad of soft, smooth filter-paper, the face of which has been moistened with a few drops of 95 per cent. alcohol. On removing the padded filter-paper, the cigarette-paper, which remains in contact with the slide and the section, is carefully stripped off, leaving the section adhering to the slide. The cigarette-paper prevents the fibers of the filter-paper from adhering to the section and marring the appearance of it.

6. Cover the section thus sticking to the slide with absolute alcohol for about thirty seconds, and drain off.

7. Flow over the section and the adjacent surface of the slide a thin solution of celloidin in equal parts of absolute alcohol and ether, draining off immediately. The film of celloidin thus formed should lie so thin as to be invisible.

8. Flood the surface of the section and of the slide with 95 per cent. alcohol, and immediately place the slide for ten seconds in water. This hardens the thin film of celloidin and prevents the section from curling and leaving the slide during the subsequent manipulations.

9. Stain with hematoxylin, or with any stain or combination of stains. The methods of staining tubercle bacilli in paraffine sections are applicable to sections obtained by this method (see p. 387).

10. Dehydrate in 95 per cent. alcohol followed by a little absolute alcohol. (The absolute alcohol should be used with care, so as not to cause loosening of the section by dissolving the celloidin.)

11. Clear with oil of origanum, and mount in balsam.

This method has been used with very satisfactory results on a wide range of pathological material. It is especially useful in the microscopical diagnosis of surgical material in connection with operations. The sticking of the section smoothly to the slide not only prevents distortion of the section by the dehydrating and clearing agents, but it also facilitates the manipulations incident to its staining and permanent mounting. By this method good smooth sections, stained, cleared, and mounted in balsam, may be obtained in the course of a few minutes after receiving the specimen.

The fixation of the material before the cutting of the section is essential to the success of the method, for if this is not done, the section will not adhere to the slide, but will adhere to the cigarette-paper in the process of pressing it on the slide. In place of the formalin, alcohol or Zenker's fluid may be used. In either case, however, the specimen must be thoroughly washed in water to remove the fixative before it can be frozen.

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Wright's Stain – Method of making.

Oliver

Add .5 gm. of sodium bicarbonate to 100 cc. of distilled water, dissolving thoroughly, and then add 1 gm. of methylene-blue (Grubler's) and heat for an hour in an Arnold sterilizer after the steam is up. After heating, allow the solution to cool. Make a 1 to 1000 solution of yellow aqueous eosin (Grubler's) and add this, while stirring, to the cooled methylene-blue solution in the proportion of 500 cc. of the eosin solution to 75 cc. of the blue solution. This should be done in a large porcelain dish. After letting the mixture stand for a few minutes, filter through one small filter paper and save the residue. The residue should then be dried in a hot-air oven and preserved.

The staining solution, ready for use, is made as follows:

Take .3 gm. of the residue, which is a greenish crystalline powder, and add it to 100 cc. of pure methylic alcohol (Merck's reagent). This should then be filtered, and to 80 cc. of the filtrate add 20 cc. of methylic alcohol. The solution is now ready for use.

To stain, add a few drops of this solution to the preparation and let stand for two minutes, then add enough distilled water to cause a slight metallic scum to form on the surface of the preparation; then let stand for two to ten minutes, and then wash the preparation in running distilled water and mount in Canada balsam.

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